Clinical Use of Bone Specific Alkaline Phosphatase of Plasma and Tumor Tissue Extract in Bone Forming Tumor

By

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Dedicate to my beloved wife

Dr. Linda, Fu Lap Kun

Acknowledgment

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ABSTRACT

With the introduction of aggressive adjuvant chemotherapy, the long-term survival rate of patients with osteosarcoma of the extremities has recently improved. As a consequence, it is now possible to evaluate some prognostic factors on a sufficiently large number of long-term survivors. Elevated levels of ALP are observed in the plasma of osteosarcoma patients. However, due to the nonspecificity of the alkaline phosphatase, the usefulness of the measurement of plasma ALP are decreased. After the introduction of Wheat germ-lectin precipitation method by Rosalki and Ying Foo in 1984, the method was widely used in many laboratory for determinating of the alkaline phosphatase isoenzyme.

The present study was divided into 2 parts. The quantitative measurement and the qualitative analysis of bone specific alkaline phosphatase in plasma and tumour tissue extract.

In the part of quantitative measurement, we contiuously monitor the level of bone specific alkaline phosphatase in the plasma of the oeteosarcoma patients, and find out that the BALP level can help the diagnosis of the disease of osteosarcoma. The effectiveness of the chemotherapy was also monitor by the measuring of the plasma ALP isoenzyme level, comparing with the clinical symptom and radiological sign. Thirdly, the result of the plasma ALP level of the osteosarcoma patients, obtained at the time of diagnosis, preoperational postoperational, and at the time of relapse was analysed the result proved that activity of plasma ALP at the time of diagnosis is related to the prognosis of the osteosarcoma patient. The higher the level of plasma bone specific ALP, the shorter the time of relapse. Moreover, level of bone specific ALP of the osteosarcoma tissue extract sampled during operation was measured. Result proved that the level of ALP isoenzyme measured in the tumor tissue extract is

significantly higher than that of the normal bone tissue. However, there is no significantly different between the patient with and without the recurrent of osteosarcoma.

In the part of qualitative analysis, plasma and tissue extract of the osteosarcoma patients was analysis. Isoelectric focusing technique was used with agarose gel as supporting media and immunological staining was used to detect the presence of the bands of different ALP isoenzymes. The result showed that the increase portion of the ALP isoenzyme of the plasma and tumor tissue extract of the osteosarcoma patients is mainly the bone specific isoenzyme. However, no abnormal band was observed by the present IEF technique.

Chapter 1
Introduction

1.1 ALKALINE PHOSPHATASE

1.1.1 Alkaline Phosphatase Isoenzyme

The human alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), E.C.3.1.3.1, ALP] comprise a spectrum of multiple molecular forms originating from various organs including liver, bone, kidney, testicular, small intestine and placenta during pregnancy. These various forms of ALP catalyze the hydrolysis of orthophosphate esters, inorganic pryophosphate and also exhibite transphosphorylation activity at optimal alkaline conditions (pH = 9 to 10) liberating the inorganic phosphate group and an alcohol (Fig. 1-1). Therefore, they constitute a system of isoenzymes (isozymes).

$$ROP + H_2O \xrightarrow{\text{orthophosphatase}} ROH + HOP$$

$$PPi + H_2O \xrightarrow{\text{pyrophosphatase}} 2P_i$$

$$ROP + RÓH \xrightarrow{\text{phosphotransferase}} ROH + RÓP$$

Fig. 1-1 General reaction that alkaline Phosphatase catalise.

The ALP isoenzymes differ from one another partly by genetic factors and by post-translational modifications. The placental, intestinal, testicular and the so-called *tissue non-specific alkaline phosphatases* (TNS-ALPs)[27, 57] differ by their primary sequences of the polypeptide chain and therefore, they are the products from different genetic loci. The first three loci are found near the end of the long arm of chromosome 2 whilst the TNS-ALP locus is found near the

end of the short arm of chromosome 1[88, 89]. The TNS-ALPs, including the skeletal, hepatic and renal enzymes, differ in the degree of post-translational modification, ***glycosylation*** (Fig. 1-2). Strictly speaking, they can only be referred as isoforms.

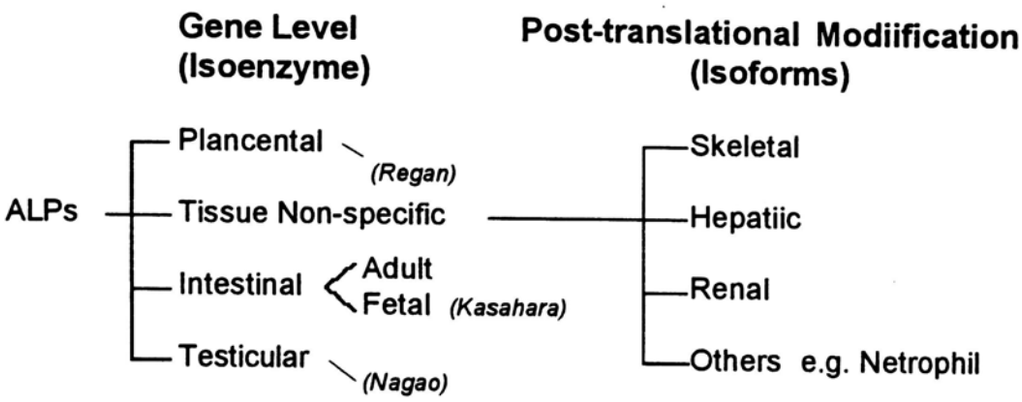

Fig 1-2 Alkaline phosphatase isoenzymes.The blacketed isoform was inappropiate expression in cancer,and act as the tumor marker.

Previous reports showed that the carbohydrates moiety of the ALP glycoproteins contains hexosamlne (e.g., N-acetylglucosamine), hexose (e.g., mannose, glucose, fucose) and sialic acid (N-acetylneuraminic acid) [25,27,31]. Detail analysis of the primary structure of the enzyme found that the liver/bone/ kidney isoenzyme comprises 507 amino acids, the intestinal 509 and the placental and the testicular isoenzyme comprises 513 amino acids.

Komoda and Sakagishi [37, 38] demonstrated that the carbohydrate side chains protect the enzyme against protease digestion. They also demonstrated that the sialic acid residue may also stabilize the native conformation of the active enzymes when subjected to heating and to pronase digestion[39]. They also demonstrated that the hexoamine of the ALP contain the sequence similar to ***"sialic acid-galactose-N-acetyl-glucosamine"*** with the penultimate galactose residue, which reveals the secretory nature of the glycoproteins.

1.1.2 The Properties of Alkaline Phosphatases

The alkaline phosphatase isoenzymes exhibit different properties when subjected to various treatments. Due to the different net molecular charges and the sizes of the molecule, the alkaline phosphatase isoenzymes exhibit different electrophoretic mobilities. Moss in 1982 found that when the five isoenzymes, which were extracted from different human tissue, are subjected to starch gel electrophoresis, two zones are frequently observed, a more anodal main zone and a low mobility, high molecular weight minor zone, which reflect exactly the properties of the major zone. The presence of minor zone result from the formation of the aggregation of other proteins and lipids with the hydrophobic domains of the ALPs, which located in the plasma membrane of the cell, leading to a larger sizes of the molecules, thus retarding migration in the starch gel. Among the five isoenzymes, placental enzymes show allelic variation resulting in three bands (Fig. 1-3). The difference in mobilities may probably be due to the different contents of their carbohydrate residue, and particularly on the presence of the negatively charged sialic acid since the desialated forms have identical isoelectric point.

Different properties are expected in the isoenzymes originating from distinct gene locus. It can be inferred from the very marked stability of the placental isoenzymes towards heat treatment at 65°C. The placental isoenzyme is completely stable at this temperature whereas the other isoenzymes, especially those TNS-isoenzyme arising from bone, kidney and liver, show different degree of inactivation by heat. The very different **thermostability** observed in the placental isoenzymes is thus thought to be due to the difference in the primary sequence of the enzyme.

Such difference in the primary sequence leads to the fact that they are

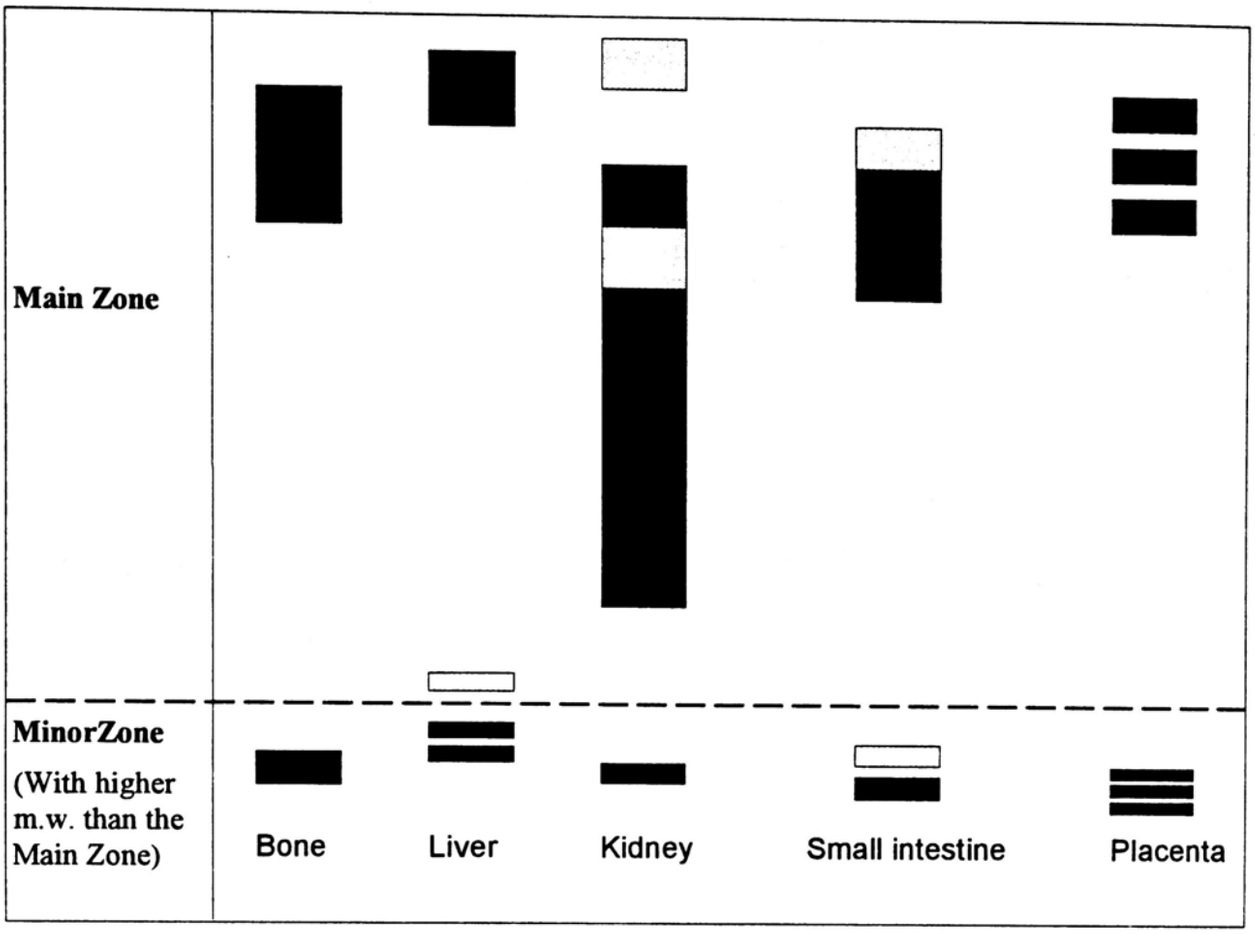

Fig. 1-3 Diagrammatic summary of differences in properties between multiple forms of human alkaline phosphatase. Some of these (e.g., placental vs. non-placental phosphatases) originate at the level of the structural gene; others are probable the result of post-translational modifications. The forms shown are based on those separated by starch-gel electrophoresis. (Moss, 1982)

also **antigenically different**. It has been observed in the placental and the non-placental isoenzymes. The sharing of some antigenic determinants between the placental and the intestinal isoform results in the cross reactions of these two isoenzymes with the whole antisera [8].

The placental enzyme also differs from the non-placental enzymes by many **catalytic properties** as they show different relative rates of hydrolysis towards various orthophosphates and pyrophosphate substrates, and greater degree of inhibition by L-phenylalanine and lesser inhibition by L-homoarginine or levamisole [1,58,80]. On the other hand, the liver and bone isoforms come from the

same genetic loci and therefore have same thermostability, antigenicity and catalytic properties. The different properties of the alkaline phosphatase isoenzymes are summarized in Table 1-1.

Table 1-1 Qualitative Properties of ALP Isoenzymes

Source	Heat Stabiliity (65°C)	Inhibtor			Antigenity
		L-Phe	L-Homo	Levam	
Liver	Liable	R	I	I	S
Bone	Liable	R	I	I	S
Plancenta	Stable	I	R	R	D
Intestine	Liable	I	R	R	D

L-Phe : L-Phenylalanine *R : Resistant* *S : Similarity*
L-Homo : L-Homoarginine *I : Inhibited* *D : Distinct*
Levam : Levamisole

1.1.3 Serum Alkaline Phosphatases

Serum from normal, healthy subjects always contain at least two isoforms of ALP, the liver and the bone ALP as detectable by electrophoresis or selective-inactivation techniques. The presence of the alkaline phosphatases, or generally speaking, any enzymes, in the serum is a result of the synthesis and release of the enzymes into the circulation by the organs involved. Their levels in the serum reflect the balance between the rate of synthesis and release into serum during cell turnover and the rate of clearance from the circulation. Increased serum levels of enzymes result from the proliferation of cells, an increase in the rate of cell turnover, passive leakage from the damaged or dying cell, or reduction of the rate of clearance. The low levels may be due to reduced synthesis, or to congenital deficiency or the presence of inherited variants of relatively low biological activity [57]. Turnover studies have shown that the

half-lives of the isoenzymes vary with a much lower value for the intestinal isoenzyme than that for the hepatic enzyme, of the order of 2 day, while the placental ALP has the longest half life of 7 day. It is possible that, as in many secretory glycoproteins, the degradation of the ALP isoenzymes is carried out by the reticuloendothelial route [19,26,56,80].

1.1.3.1 Placental Alkaline Phosphatase

Placental alkaline phosphatase, derived from syntrophoblasts, becomes detectable in serum of pregnant women in 16th to 20th weeks of gestation and increase progressively up to onset of labour. They disappear within 3 to 6 days of the delivery of the placenta. Complications of pregnancy such as hypertension, pre-eclampsia, eclampsia and in the cases of threatened abortion are always reflected in elevated serum level of placental ALP [34]. High level of serum placental ALP were sometimes found in patients with cholestatic liver diseases[17]. The inability of the placental ALP to cross the placenta renders the amount of this enzyme in the serum of newborn infant undetectable

1.1.3.2 Intestinal Alkaline Phosphatase

The isoenzyme analysis of serum has shown that small amount of intestinal ALP can be found in about 25% of normal sera, most of which are more probable to the subjects of B- or O-blood group who are secretor-positive. This may be explained by the recent work [57] which has shown to that intestinal ALP is bound by the erythrocyte of the group A but a lesser degree by those of the group B or O. The low level of this isoenzyme in normal serum is accounted for by its clearance from the circulation at a significantly higher rate than the other isoenzymes [17]. After each meal, this isoenzyme has been shown to increase in those whose serum shows detectable isoenzyme [40]. Pathologic increases of the

serum intestinal ALP are observed in various diseases of the digestive tract, liver cirrhosis, intrahepatic cholestasis and those patients undergoing chronic hemodialysis [16,36,77,82].

1.1.3.4 Skeletal Alkalne Phosphatase

The bone and liver ALPs are invariably present in serum. The bone-specific alkaline phosphatase is synthesized and secreted into the serum by the bone forming cells of the skeletal organ - osteoblasts. The bone-specific ALP has long been considered as an index of osteoblastic activity. The normal serum level of bone-specific ALP is dependent on age at about 4 times higher in puberty than in adult. This physiological increase in puberty reflects the higher osteoblastic activity which is essential for adolescent growth spurt of bone. After the growth spurt, the serum level of bone-specific ALP drops to a low level, about 50 IU/L and shows a steady rise throughout the life. Pathologic increase of serum bone specific ALP are observed in various malignant tumours possessing osteoblastic activity such as osteogenic sarcoma and secondary deposits of carcinoma in bone, Paget's disease of bone, osteomalacia and rickets resulting from malnutrition, malabsorption or renal failure[85]. Increase in osteoblastic activity is also observed in bone fracture, thereby, increasing serum level of bone-specific alkaline phosphatase. (Fig. 1-4)

1.1.3.5 Hepatic Alkaline Phosphatase

As shown in Fig. 1-4, normal serum level of the hepatic ALP increases steadily throughout life. The hepatic ALP is found in high concentration in the hepatobiliary tract, therefore, the diseases of intra- or extrahepatic cholestasis resulting from, for instance, advance primary liver cancer, carcinoma of the pancreatic head etc., always leads to the high level of the hepatic ALP in serum.

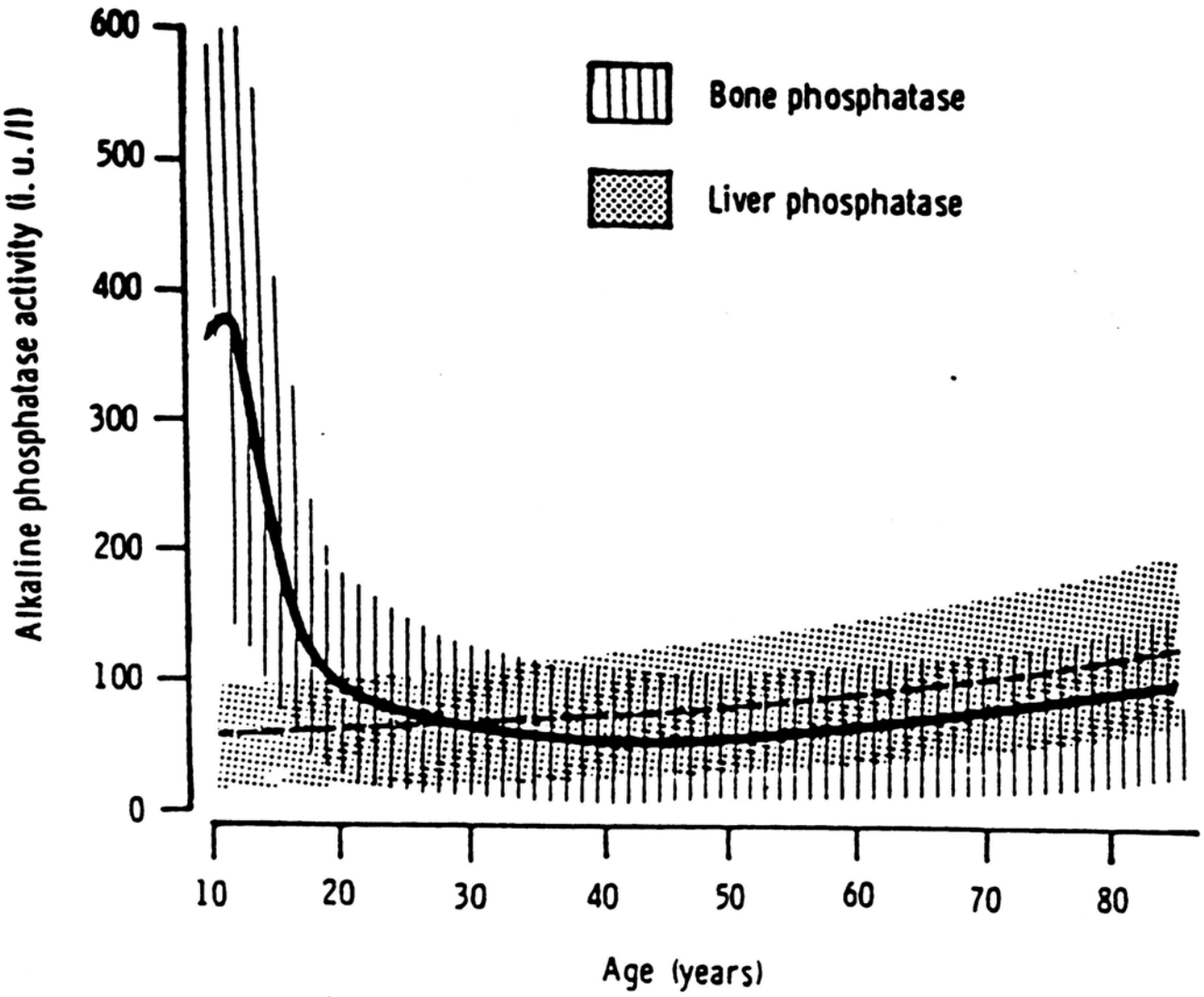

Fig. 1-4 Changes in the relative activities of bone and liver alkaline Phosphatase in human serum with age .

1.1.3.3 Renal Alkaline Phosphatase

Renal alkaline phosphatase is present in particularly high concentration in renal tubules and is absent in serum in normal healthy subjects [29]. Renal diseases also rarely lead to serum renal isoform but it has been observed during rejection of the renal transplant.

1.1.3.6 Miscellaneous Alkaline Phosphatase

The sera from patients with hepatobiliary disease show minor phosphatase component, with relatively higher molecular mass, form a low mobility band in starch gel or polyacrylamide gel electrophoresis. While in non-sieving media

such as cellulose acetate, this band, because of the higher molecular charge, migrates more rapidly towards the anode than the main liver band. The band has been termed the "fast liver" or ''α_1 liver" fraction [57]. This kind of ALP has been shown to be the hepatic enzyme in nature associating with fragments of cell membrane from biliary tract cells or hepatocytes. It has been termed biliary alkaline phosphatase, releasing into the blood as a result of cell damage [15]. The presence of the fast liver band is a valuable evidence of the disease of extrahepatic obstruction.

Another kind of ALP is found in sera from many patients suffering from malignant diseases. The first of such cancer-related isoenzymes to be discovered, the Regan isoenzyme, is essentially identical to normal placental alkaline phosphatase, even reflecting that isoenzyme's allelic variation. The identification of another PLAP-like isoenzyme, the Nagao isoenzyme, is found in testis and thymus and some other tissue. The overall incidence of these two isoenzymes is about 5-15%, while the incidence as high as 60% may be found in germcell tumor[57]. Reports have demonstrated that the serum samples of some patients with hepatoma show a more anodal band than the main intestine ALP in gel media. This band is termed the Kasahara isoenzyme which has the catalytic properties typical of intestinal phosphatase. However, unlike adult intestinal phosphatase, the Kasahara isoenzyme has terminal silialic acid residue and has a more rapid anodal migration on electrophoresis.

These cancer-derived isoenzymes may be indistinguishable from the normal placental ALP, which can be accounted for by depression of the placental isoenzyme gene by the malignant cells. However some differences between these cancer-related ALP and their normal analogues presumably arise by post-translational modifications that may be specified to cancer cells. Altered patterns of glycosylation may exist in cancer cell. Therefore, these isoenzyme may

sometimes be detected at pre-malignant conditions [59]. The properties of the three cancer derived ALP variants, the Regan, Nagao and Kasahara isoenzymes, were summarized in the following table.

Table 1-2 Three cancer-derived ALP variants that are presumed to originate from inappropriate expression of Alkaline Phosphatase-Determining genes

Normal isoenzyme	Tumour variant	Characteristic properties
Term-placental ALP	*Regan isoenzyme "PLAP"*	Heat-stable, reacts with anti-placental ALP antisera, L-Phe sensitive, variable electrophoretic mobility as PALP
Testicular ALP	*Nagao isoenzyme "PLAP-like"*	Heat-stable, differ with PALP or Regan isoenzyme with some monoclonal antibodies, L-Phe sensitive
Fetal-intestinal ALP	*Kasarahara isoenzyme*	Heat stable, react with anti-intestinal antisera, binds to Reactive Yellow 13, L-Phe & L-Leu sensitive, sialylated, rapid anodal electrophoretic mobility

1.1.4 Problems in Discriminating the Skeletal and Hepatic Alkaline Phosphatase in Serum

Elevated serum levels of ALP are observed in many disease status, as previously described. Therefore, identification of the ALP isoforms that contribute to the raised serum level is important in diagnosis of many diseases. Identification of the placental and intestinal isoenzymes from the TNSALPs is quite straight forwards by exploitation of their thermostability and their different catalytic properties towards various substrates (Table 1-1). However, the distinction between hepatic and skeletal isoforms is not easily achieved due to their similar antigenicity, thermostability and catalytic properties towards various substrates and inhibitors, probably as a result of the translation from the same

genetic locus. The great majority of the requests for the isoform analysis also aims at distinguishing the bone and liver as alternative or coexisting sources of the elevated ALP activity in serum.

1.1.5 Quantitative measure of the Bone-Specific Alkaline Phosphatase —— *Wheat Germ Lectin Precipitation*

Rosalki and Foo in 1984 first introduced a method to quantify serum bone-specific ALP by the use of wheat germ lectin[63]. Lectins are highly specific, carbohydrate binding proteins of plant origin, the wheat germ lectin from *Triticum vulgaris* specifically binds to the N-acetylglucosamine residue and its derivatives, e.g., N,N'diacetylchitobiose[3,9] of the carbohydrates side chains of many glycoproteins. After binding to the sugars, its large molecular weight leads to the precipitation of the large lectin-glycoproteins complex. Since ALP isoenzymes are N-acetylglucosamine-containing glycoproteins, they have been demonstrated to be capable of being bound by wheat germ lectin with the skeletal isoform being preferentially bound. The sugar composition of skeletal ALP has higher content of N-acetylglucosamine than the hepatic, intestinal or placental isoform. Therefore, when the other three isoforms coexisting with the skeletal isoform, the very strong binding between the skeletal ALP with the wheat germ lectin rendering the percentage binding between that of the hepatic intestinal and placental isoform insignificant.

The method described by Rosalki and Foo[63] involved a precipitation of the skeletal isoform with wheat germ lectin and reconstituted the precipitate for the skeletal ALP assay, together with the assay of total ALP in plasma sample, the hepatic isoform being calculated from the difference between the two values. The liver fraction, or the supernatant, comprises mainly the hepatic isoform and some small amount of intestine and placental ALP isoenzyme. However,

the activity of the intestine and placental ALP isoenzyme is too low to significantly interfere the result. They also demonstrated that around a quarter of the high-molecular weight biliary ALP would be co-precipitated as the skeletal fraction. However, this could be avoided by the pretreatment of the sample with Triton X-100. The function of Triton X-100 surfactant is to solubilize the membrane fragment of the biliary ALP, thus converting them into the hepatic isoform. On the contrary, the pretreatment of Triton X-100 will not affect the differentiation between the bone and liver isoenzymes.

In addition, Rosalki and Foo reported that the skeletal isoform precipitated was only 80% of the total skeletal isoform present. This has been disproved by W. Behr and J. Barnert[6] by their demonstration of complete precipitation of ALP activity (98-99%) by using cord sera which are shown to contain only skeletal ALP activity. The partial precipitation of the skeletal isoform by Rosalki and Foo may be due to the batch variation of the wheat germ lectin they purchased, and the inaccuracy of the method of heat inactivation electrophoresis.

Futhermore, skeletal and hepatic ALP of the butanol or aqouse extract in the bone tissue were less effectively differentiated by wheat-germ lectin. The isoenzyme isolated from liver and placenta and the one extracted from bone are all similarly bound by the wheat-germ lectin. Whereas hepatobiliary and placental ALP in plasma are obviously not precipitated. It may be presumably because of alteration in surface carbohydrate during extraction[52], or possibly because membrane-localized glycosyl-transferases modify the enzyme molecule as it passes into the intravasal space[46-48]. However, the extract from the bone contain mainly the skeletal isoenzyme, the percentage of the liver and other portion in the bone extract is negligible, so we can presume that the activity of skeletal isoenzyme approximately equals the total ALP activity in the bone extract.

1.1.6 Qualitative Detection of ALP isoenzymes—— *Electrophorisis*

Zone electrophoresis followed by staining for enzymatic activity, usually with α-naphthyl phosphate as substrate and with a diazonium salt to detect the liberation of α-naphthol, is the most useful single technique for the qualitative detection of multiform of the alkaline phosphatase. Electrophoresis in gel media such as the starch or polyacrylamide, or the seiving-gel media such as the cellulose acetste or agarose gel were used. However, the incomplete separation of the skeletal and hepatic isoforms makes the densitometric quantification of the enzymes difficult [52,73]. Afterall, some simple selective inactivation method may leads to a more distnct seperation between these isoenzyme. The simple heat inactivation assay will give semi-quantitative estimates of the bone-specific ALP, in which a residue activity of less than 20% after heat treatment at 56°C for 10 minutes reflects the presence of skeletal isoform in a larger proportion[83]. The improved sequential heat inactivation method at 56°C will give quantitative estimates of the bone-specific ALP but it is too tedious to perform and the calculation involved is quite complicated; therefore, it is impractical for large scale, routine analysis of the samples in clinical laboratories [3]. Rosalki and Foo[62] also described another method for the separation of the skeletal isoform from the non-skeletal isoforms, namely affinity electrophoresis, by presoaking the cellulose acetate membrane with buffer including wheat germ lectin before electrophoresis. This method had been demonstrated to separate the skeletal isoform from the hepatic isoform, which could not be resolved completely in conventional unmodified method. Moreover, the pretreatment of the sample serum with the neuraminidase[64,69] was also proved to give a better seperation of the two isoenzyme.

The introduction of Isoelectric Focusing technique (IEF) improve the resolution of the different bands of the ALP isoenzyme. J. Griffiths and J. Black

in 1987 reported a reliable and reproducable IEF technique[27], using 1mm thick agarose mixing with ampholytes of pH 3.5-9.5 as suporting media, using electrode of pH 3-10, performing IEF at 15W constant power for 35 minutes and using 1-naphthy phosphate and diazonium salt as substrate. They found 12 sharp and clear bands of different ALP isoenzyme with pI 3.01 to 4.86. Some other authors[27,74,90], used polyacrylamide gel as supporting media or 5-bromo-4-chloro-3-indoxyl phosphate as substrate. The different supporting media, different substrate used and different resulting bands found was summerized in the followering table 1-3.

Table 1-3 Different IEF criteria used by previous researchers and the results found.

Author	Gel film	Substrate & Stainrange	pH range	No. of bands	Isoelectric points (pI)			
					Liver	*Bone*	*Placent.*	*Intest.*
Griffiths & Black *1987*	Agarose gel	1-naphthyl phosphate with 4-aminodiphenyl-amine diazonium salt	3.5-9.5	12	3.65	4.21	4.65	4.86
Blum-Stolnick *1983*	polyacryl-amide gel	1-naphthyl phosphate monosodium salt and Variamin Blue with copper sulfate	3.0-9.0	7	4.3	4.5	3.6	4.9
Sinha *1986*	polyacryl-amide gel	5-bromo-4-chloro-3-indoxyl phosphate	3.5-6.0	10	4.9	5.09	4.73	

Electrophoresis allows us to see each of the isoenzyme, and especially the unusual isoenzyme. For instance, P.K. Sinha[74] in 1986 find out two distinct band with pI spetra of 4.95 and 5.90 in patients with Paget's disease. This show the great potential of the use of qualitative analysis of the alkaline phosphatase as a tool to discover the unusal isoenzyme of the individual patient group, probabily to detect or diagnose the presence of malignant disease.

On the other hands, extract of the tumour tissue has also been analysed by the method of electrophoresis. The number and intensity of ALP bands separated by electrophoresis depends on the difference in carbohydrates and siaic acid content and vary considerably according to the methods used for extraction from tissue [23,44,52]. Many methods for extraction have been reported by the previous authors and some of them are summerized in the following table.

Table 1-4 Qualitative analysis of alkaline phosphatase isoenzyme in the tumour extract

Author	Buffer used	Media for extraction	Method used	Results
K. Masuhara (1987)	borate buffer	n-butanol	Chromatography & electrophoresis	a band with m.w. 80000 which similar to the bone ALP
H.J.M. Cleve (1984)	Tris buffer	n-butanol	IEF	4 band of isoenzyme of ALP but unable to qualitate thebone & liver isoform by scanning
A.M. Levine (1979)	amino,2-methyl-1-propanol	distilled water	electrophoresis and IEF	Osteosarcoma tissue with activity = 2.02 umol/min/mg (202 U/gm)
L. Kadlecova (1975)	0.1 M borate	redistilled water	electrophoresis	Thermostability and the pH stability was reported

1.2 OSTEOSARCOMA

1.2.1 Definition

The current international definitions of Osteosarcoma base on the presence of tumour osteoid or immature bone, is unsatisfactory. With the development of Immunohistochemical staining the osteosarcoma is divided into four types, the osteoblastic, chondroblastic, fibroblastic and telangiectatic types. They all revealed a significant degree of alkaline phosphatase activity [9,54,68,81,86]. While the chondrosarcorma and the fibrosarcoma can be separated from the chondroblastic and fibroblastic osteosarcoma by their absence of alkaline phosphatase staining. They are summarised in the following table.

Table 1-5 *Immunohistochemical Findings in Different Types of Osteosarcoma in Comparison with other Chondroid Tissues*

	Osteosarcoma				Chondrosarcoma	fibrosarcoma
	Osteo.	Chondro.	Fibro.	Telang.		
ALPase	++	+++	++	++	-	-

Osteo.; Osteoblastic type, Chondro.; Chondroblastic type, Fibro.; Fibroblastic type, Telang.; Telangiectatic type, +++; markedly positive, ++; moderately positive, +; slightly positive, -; negative.

1.2.2 Epidemiology and Statistics

Next to plasma cell myeloma, osteogenic sarcoma is the most common primary malignant tumour of the bone [19,40,86]. The estimate incidence of this tumour is 1.7 cases per million per year in United States [40], 1.1 cases per million per year in Malaysia, and 2.3 cases per million per year in Chinese and Indian

populations [42]. Although it can occur in any age group, 50% of the cases occur in the second decade. Males are more frequently than females; the ratio of incidence is 1.6 to 1.0. The median presentation in females is two years earlier than males. This is probably related to the earlier growth spur in females [35].

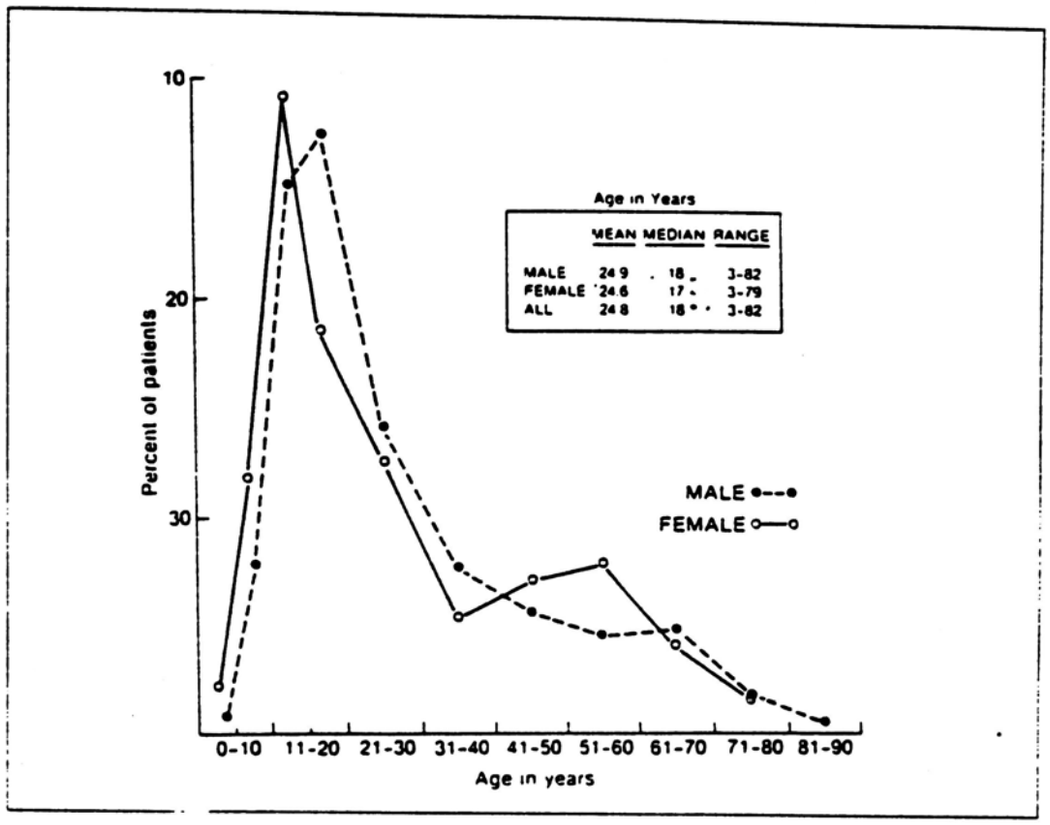

Fig. 1-4 Age and sex distribution in 1095 patients with osteosarcoma diagnosed in Memorial Sloan-Kettering Cancer Center 1921 through 1979.

The appendicular skeleton is most frequently involved and approximately 51% of the tumour occur about the knee. On the other hand, osteosarcoma more readily in the areas with the greatest number of active osteoblastic cells such as the metaphases of the long bones [12,35,40,86].

1.2.3 Clinical Presentation

Usually the young patients gives a short history of increasing pain and swelling in the lower limb that may following trauma. Most centres now agree

that trauma brings the patients' attention to preexisting tumour. A history of limping or limb protection may be reported. A firm fusiform eccentric swelling may be found on physical examination and it may be relatively painless to palpation. Temperature and overlying skin may be increasing in the patients with large tumour.

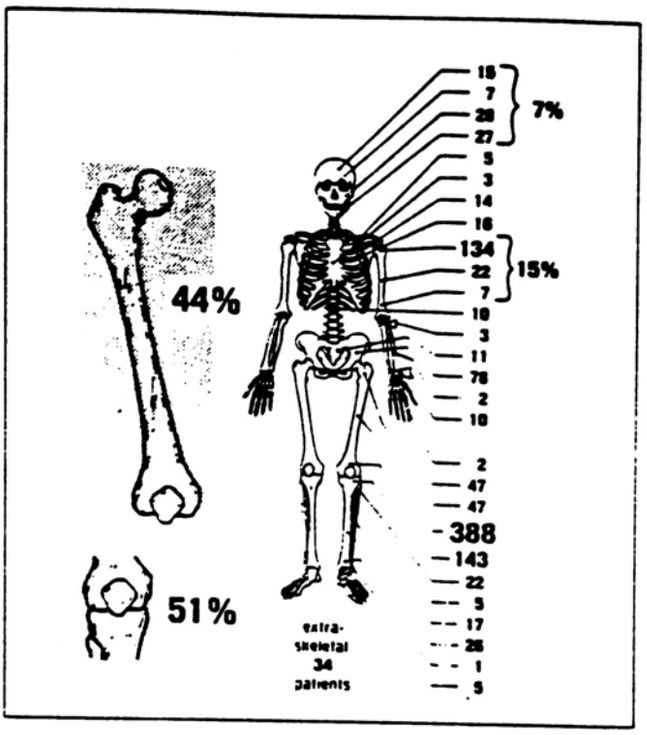

Fig. 1-5 Skeletal distribution in 1095 osteosarcoma patients diagnosed at Memorial Sloan-Kettering Cancer Center 1921 through 1979.

1.2.4 Radiographic finding

Radiographically osteogenic sarcoma may be lytic or sclerotic base on the amount of the tumour bone production [40,72,86]. Classically, the tumour is situated in the metaphysis of the long bone, the characteristic radiographic changes included;

1) Destruction of pre-existence of cortical or medullary bone, the sun-ray burst.
2) Calcification and bone production.
3) Periosteal reaction with new bone formation, the Codman's triangle.

However, neither of these signs is pathogonomonic for malignancy.

The distinctive radiographic patterns were found by von Ronnen [56]. The most frequent was the mixed type, which demonstrated mixed patterns of bone formation and destruction, and accounts for 75%, On the other hands, the

purely sclerotic type was 14% and the purely lytic type was 11%. However, no significant difference in survival were noted among the various type.

Computerized Tomography (CT) and Magnetic Resonance Image (MRI) are valuable methods of finding the osseous and soft-tissue extent of the tumour and determining their relationship to major neurovascular structures and adjacent joints [72,86]. Radionuclide bone scanning with technetium 99m methylene diphosphonate (^{99m}TcMDP) assists in the detection of osseous metastatic lesions. Angiography is excellent in detect of local reccurent soft-tissue disease.

1.2.5 Staging of Musculoskeletal Neoplasms

It has recently been adapted by the American Joint Committee Task Force on Bone Tumour and proposed by them to the International Union Against Cancer (IUAC) for international usage. It is based upon the histological grading (G), anatomy site (T), presence or absence of metastasis (M) [20]. The system is summarized in the following table:

Table 1-6 Stages of Musculoskeletal Lesions

Stage	Description	Grade(G)	Site(T)	Metastasis(M)	Lodwick
Begnine					
1	latent	G_0	T_0	M_0	Ia
2	active	G_0	T_0	M_0	Ib
3	aggressive	G_0	T_{0-2}	M_{0-1}	Ic
Malignant					
I*	low grade without metastasis	G_1	T_{1-2}	M_0	II
II*	high grade without metastasis	G_2	T_{1-2}	M_0	III
III *	low/high grade with metastasis	G_{1-2}	T_{1-2}	M_1	II-III

** with 2 subgrades, A=Intracompartment, B=Extracompartmental*

The system was trials by the Musculoskeletal Tumour Society and was shown to practical, reproducible and valuable in surgical planing [7,21]. However, the usefulness was still limited for assessing prognosis for either groups of lesions or individual cases [86].

1.2.6 Treatment of osteosarcoma

In the past, amputation of the limb was the only treatment available [60,78]. During that period, the five-year survival rate was at best 20% [51]. Recently, advances in chemotherapy and orthopaedic surgical techniques have significantly change the outlook for patients with osteosarcoma [86]. Limb salvage operation with allografts, prothesis or rotationplasty technique plus the utilization of pre-operational and post-operational chemotherapy [32,50] can improve the survival rate to the best 93%[11,40,55,65,86]. Even in Rizzoli, where the prognosis has been worse than the other countries, the five-year survivial rate was improved from 10% to 45% [70].

1.2.6.1.Chemotherapy in Prince of Wales Hospital

Three protocol of adjuvant chemotherapy was used in the Oncology Department of PWH. The usual regime is protocol A, (Table 1-7) which contains three drugs;

1) High dose of methotrexate (MTX), which is the inhibitor of folic acid and therefore inhibit the synthesis of DNA and thymidine monophosphate.

2) Adramycin (Adr) and

3) Cisplatinum (Cis), which are the inhibitor of mitotic division.

Table 1-7 Regime A chemotherapy used in the Oncology Department of PWH for the osteosarcoma patients

Date	Drugs used	Monitor in each course
Week 1	Cis + Adr	CBP, LFT, LDH, CrCl, and
Week 3	MTX	X-ray of the tumour site
Week 4	MTX	
Week 5	MTX	CXR every month
Week 6	MTX	CT before operation
Week 7	Cis + Adr	
Week 10	Operation	
Week 13	Cis + Adr	
Week 15	MTX	
Week 16	MTX	
Week 17	Cis + Adr	
Week 19	MTX	
Week 20	MTX	

After week 20, if favourable, repeat the cycle from week 13 to 20. While in unfavourable cases, change to other regimes.

The oncologists suggested that 80% - 95% of patients presumably had already systemic metastasis at the time of original resection[65], therefore, post-operative chemotherapy can significantly improve the survival rate. On the other hand, the pre-operative chemotherapy can decrease the tumor size and improve the ability of limb salvage operation.

1.3 PLASMA AND TISSUE ALKALINE PHOSPHATASE IN NORMAL AND NEOPLASTIC CONDITION

1.3.1 Normal values of plasma alkaline phosphatase

The determination of plasma alkaline phosphatase values can be studies by different assay methods. Moreover, assay plasma levels may be expressed in various units, using different substrates and at varies temperatures. Different methods and unit used were sumerized in the following table.

Table 1-8 Normal plasma alkaline phosphatase values in adults and children (Units/dl at 37°C)

	Adults		Children	
Methiod	Range	Average	Range	Average
International Unit (IU)	31-82*	56.5	72-247*	159.5
Bondansky	1.5-4	2.6	5-14	7.7
King Amstrong	4-10	8.0	10-25	20.0

The International Unit of enzyme is micromloes per minutes, and the corresponding activity concentration is in unit per liter.

** The substrate and the IU values are expressed in mmol/P-Nitrophenyl phosphate/ min of ml serum at 37°C.*

After the introduction of Wheat germ-lectin percipitation method by Rosalki and Ying Foo in 1984, the method was widly used in many laboratory for determinating of the alkaline phosphatase isoenzyme. The table 1-5 sumerized the normal value of the alkaline phosphates isoenzyme by using the wheat germ-lectin percipitation method. The unit used in the present experiment for the alkaline phosphatase and its isoenzyme is the International Unit (IU), and the upper limite of bone specific ALP for adult is 56 IU/L, for adolescence with age 12 to 16 yrs is 165 IU/L.

Table 1-9 Normal value of alkaline phosphatase isoenzyme determined by wheat germ-lectin percipitation method, using international unit (IU).

Author	Age group	Mean \ Range (S.D.) of ALP Isoenzyme		
		Total	*Bone*	*Liver*
Rosalki & Foo 1984	Adult (Male)	108 (22)	81 (22)	27 (9.4)
	(Female)	89 (19)	57 (11)	33 (8.2)
Werner Behr 1986	6 day-14 yr	189-662 (130.9)	120-544 (135.9)	
	Adult (Male)	50-137 (17.4)	27-96 (14.5)	
	(Female)	53-155 (29.6)	32-102 (18.4)	
PWH main lab.	12 yrs to 16 yrs	74 - 235	36 - 165	
	> 16 yrs	40 -136	23 - 56	
K.S. Leung 1992	Adult (Male)		39.76 (16.68)	
	(Female)		31.36 (12.41)	

PWH = Prince of Wales Hospital Maim Laboratory

The previous study in our laboratory [49] main component of isoenzyme in children is bone specific isoenzyme, it declines rapidly in aldolesence. While the liver portion of the alkaline phosphatase was stadily increase with age (Figure. 1-6 a,b) below.

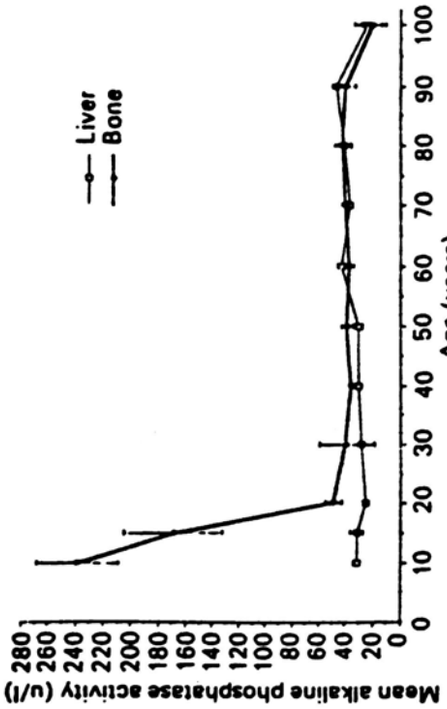

Fig. 1
Mean levels of bone and liver alkaline phosphatase activity in the plasma of normal adolescent and adult males (error bar = SEM).

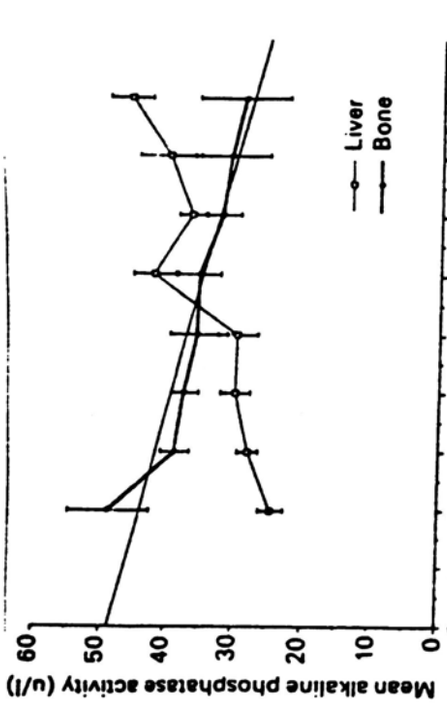

Fig. 2
Mean levels of bone and liver alkaline phosphatase activity in the plasma of normal adult males. The regression line represents the bone-specific enzyme activity ($y = 48.624 - 0.22889x$ $R^2 = 0.845$).

1.3.2 Clinical use of elevated plasma & tissue alkaline phosphatase level in neoplastic conditions

McKenna and associates pointed out the possible prognostic value of the serum alkaline phosphatase level [54]. This and other studies firmly established that these values are helpful in determining prognosis. Postoperative, post-chemotherapy follow-up should include alkaline phosphatase determination, since the elevation of ALP activity herald the presence of residual, reactivate, and spreading osteogenic sarcoma. In other words, it detect the recurrence or metastasis of the disease. Moreover, plasma alkaline phosphatase level can also be used to monitore the effectiveness of chemotherapy.

1.3.2.1 Helping the Diagnosis of the Osteosarcoma

In general, there is fairly good correspondence between the activity of alkaline phosphatese and the degree of the osteoblastic activity within the osteosarcoma. Ultrastructure localization found out that alkaline phosphatase was located over the plasma membrane and associated subplasmalemmal vesicle, and vacuoles of osteogenic sarcoma cell [4,84]. Abnomally high level of the plasma alkaline phosphatase in patients with osteosarcoma. Previous works of many authors reported that 40% to 80% of the patients with osteosarcoma has elevated level of alkaline phosphatase, during the time of diagnosis[5,54,70,79,81]. Morover, previous work of our laboratory[49] reported that all of the 15 osteosarcoma patients had elevated plasma alkaline phosphatase level at the time of diagnosis. Alan M. Levine et al. in 1979 found out that the alkaline phosphatase level in the osteosarcoma tissue extract was very high, with mean value of 0.2 umol/ min/mg tissue (200 U/gm tissue), comparing with that of the normal cortical bone[44]. These showed that plasma and tissue alkaline phosphatase can help diagnosis of osteosarcoma, especially in the clinically boderline or non-characteristic cases.

1.3.2.2 Monitoring the effect of chemotherapy

The reduction of elevated plasma alkaline phosphatase values following chemotherapy is a valuable guild to administration of therapy (Fig. 1-8). It corelated to the decrease of osteoblastic activity of the tumor, reduction of the tumor size, and improvement of the clinical presentation. The return of abnormal level signals the recrudescence of active disease, and the increase of doses of adminstration of the chemotherapy, if toxicity permits, should be undertaken forthwith [67,79].

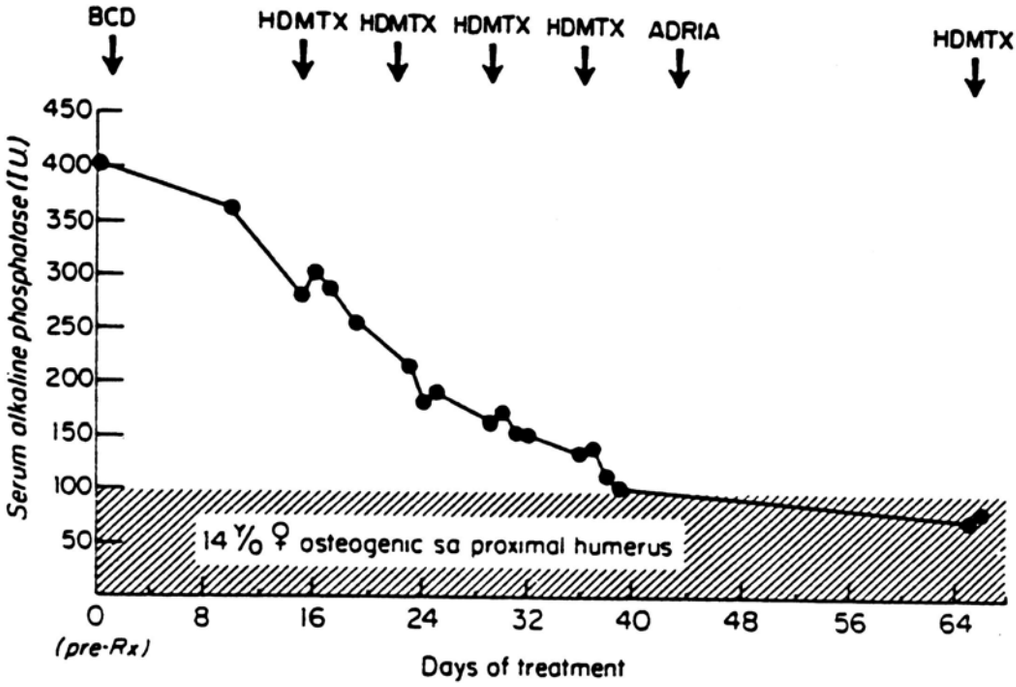

Figure 1-7 The effect of neoadjuvant chemotherapy on serum alkaline phosphatase values in a patient with proximal humeral osteogenic sarcoma. The decine of elevated values to normal levels (shaded zone) signifies the sarcoma is responding favorably to chemotheraphy. (From Dr. G. Rosen)

1.3.2.3 Predicting the clinical course

With the introduction of aggressive adjuvant chemotherapy, the long-term survival rate of patients with osteosarcoma of the extremities has recently improved from approximately 10-20% [14,45,75] to 40-70% [5,10,66]. As a consequence, it is now possible to evaluate some prognostic factors on a sufficiently large number of long-term survivors.

Pretreatment evaluation

The results concerning the prognosis significance of the pretreatment plasma alkaline phosphatase level have been contradictory in patients with osteosarcoma. Thorpe et ai.[79] from the National Cancer Institute at Bethesada (NCI) conclude that pretreatment plasma ALP level could be a prognostic factor in osteosarcoma: the higher the ALP levels, the higher the rate of relapse. This result was supported by G. Bacci in Rizzoli [5], which a larger group of 163 osteosarcoma patients. However, the result of UCLA [18] reached the conclusion that there is no direct relation between the elevated plasma ALP level, tumour recurrence and prognosis. The result were summerized in the following table.

Table 1-10 Relationship between the level of serum ALP preoperative and postoperative to the prognosis of osteosarcoma

Authors	Preoperative ALP Level	No. of patient	Percentage of relapsed	Time to recurrent
G.Bacci (1987)	Normal	42	19 %	17.1 months
	Elevated	121	64 %	10.8 months
W.P.Thorpe (1979)	Normal	13	31 %	33+11 wks
	Elevated	17	71 %	19+3 wks
P.E.Scranton (1975)	Normal	22	46 %	Not reported
	Elevated	16	81 %	
Authors	**Postoperative ALP Level**	**No. of patient**	**Percentage of relapsed**	**Time to recurrent**
G.Bacci (1987)	Normal	89	55 %	13.2 months
	Elevated	32	90 %	6.0 months
W.P.Thorpe (1979)	Normal	Result are not significantly different		
	Elevated			
P.E.Scranton (1975)	Normal	-	Not reported	-
	Elevated	-	100 %	

Postoperative evaluation and correlation with prognosis

G. Bacci also point out that there is the existence of a correlation between the postoperative serum ALP levels and the prognosis. In patients with elevated serum ALP level at presentation but drop back to normal after surgical removal of the tumour, the rate of recurrent was lower than that of the patients who lacks of this response. However, due to the different methods of operative treatment, different scheme of chemotherapy and thefalse increase due to the Kuntscher rod or the autograft, the relationship of the postoperative serum ALP level was not so significant as that of the pretreatment serum ALP values. Table 1-10 on the previous page.

On the other hand, Alan M. Levine[44] reported that with the activity of the sarcoma tissue extract higher than 0.6 umol/min/mg tissue (600 U/gm tissue), therate of recurrent was significantly higher than that of the patient with lower activity of the tumor tissue extract.

Analysis of serum ALP at the time of relapse

The studies of many previous authors proved that the serum ALP activity raised to an abnormal level at the time of recurrent or metastasis[53]. Moreover, the raise of serum ALP preceded by several weeks the appearance of symptom and clinical sign, and before the detection of metastasis by x-ray. In the study of G. Bacci[5] for example, 31 out of 52 patients who developed metastasis during the adjuvant chemotherapy, had an abnormally high level of serum ALP. While the rise of the serum ALP preceeding any x-ray demonstration by 4 to 12 weeks, and preceeding any clinical sign by 8 to 16 weeks. Therefore, serum ALP was included in many institudes during the period of follow-up.

M.W. J. Davie in 1991 gave a very typical case report on the use of serum alkaline phosphatase to monitor the period of follow-up of a 65-year-old woman

with Paget's disease[15]. He found that the serum alkaline phosphatase response to the removal of the tumor by a sharp reduce of the activity, and rose briskly preceed the recurrent of the tumour. This proved that the level of serum alkaline phosphatase is a good marker of the osteoblastic activity of the bone forming tumour.

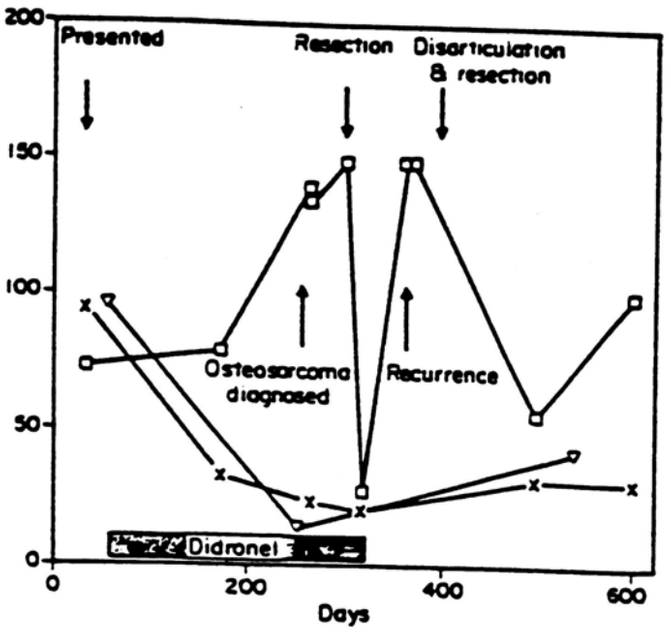

Figure 1-8 Relationships among serum ALP during the treatment and progression of osteosarcoma.

1.3.3 Qualitative measurement of ALP in plasma and tissue extract of osteosarcoma patient

It has been noted that some sorts of tumour, such as the hepatoms, testicular seminoma or pulmonary carcinoma etc., has specific isoform of alkaline phosphatase which formed by the transglycosilation probably by the tumour cells. For instance, the Regan, Nagasa and Krada isoforms of ALP. Kensaka Masukara in 1987 [52] purified the bone specific ALP from the human osteosarcoma tissue. The procedure including the chromatography and electrophoresis. The purified enzyme is a glycoprotein with molecular weight [37] of 80,000 and is characteristically inhited by modest heat (56° C, 30 min.) and L-homoarginine but not by L-phenylalanine. These properties was similar to that of the bone specific isoenzyme reported previously.

Therefore qualitative analysis of the plasma or tumour tissue extract of the osteosarcoma patient is very important and holpfully it may give a specific band which can help diagnosis of this disease.

1.4 AIM AND SCOPE OF THE PRESENT DISSERATION

Increase level of serum alkaline phosphatase has long been referred to as having increased osteoblastic activities in bone. It appears to be the only laboratory test for diagnosing and monitering the disease of bone forming tumour, especially the osteosarcoma. In the present study, level of bone-specific isoenzyme of alkaline phosphatase, in plasma and extract of the tumour tissue, was measured to see whether it could act as a marker of the bone forming tumour.

The present study was divided into 2 parts.The quantitative measurement and the qualititative analysis of bone specific alkaline phosphatase in plasma and tumour tissue extract.

In the part of quantitative measurement, we contiuously monitor the level of bone specific alkaline phosphatase in the plasma of the oeteosarcoma patients, and find out whether the BALP level can help the diagnosis of the disease of osteosarcoma. The effectiveness of the chemotheraphy was also monitor by the measuring of the plasma ALP isoenzyme level, comparing with the clinical symptom and radiological sign. Thirdly, the result of the plasma ALP level of the osteosarcoma patients, obtainded at the time of diagnosis, preoperational postoperational, and at the time of relapse was analysed and see wether the activity is related to the prognosis of the osteosarcoma patient, the length of period of the recurrence of the diseased. Moreover, level of bone specific ALP of the osteosarcoma tissue extract sampled during operation was measured. Relation between the level of ALP isoenzyme measured in the tumor tissue extract and the prognosis of the osteosarcoma patient was also studied.

In the part of qualitative analysis, plasma and tissue extract of the osteosarcoma patients was analysis. Isoelectric focusing technique was used

which allows us to see each of the isoenzyme, and especially the unusual isoenzyme. This show the great potential of the use of the alkaline phosphatase as a tool to discover the unusal isoenzyme of the individual patient group, which can act as the tumor marker or probabily to detect or diagnose the presence of malignant disease.

Chapter 2
Materials and Methods

2.1 DIFERENT GROUPS OF PATIENTS

In the period of October 1991 to April 1993, 20 patients with the desease of osteosarcoma; 34 patients with benign bone tumour and 26 patients with malignant tumour that migrated to the skeletal system were recorded when they were admitted or refered to the Department of Orthopaedics and Traumatology, Prince of Wales Hospital. They were divided into 3 groups, namely the Osteosarcoma group, the Benign group and the Metastasis group respectively. The patients were monitor until the April 1994. The mean follow up time of Osteosarcoma group was 17.8 months, 27.6 months for the Benign group and 18.8 months for the Metastasis group. The details of patients of the different group was summerized in the following table.

Tabbe 2.1 Different groups of patients collected in PWH

Patient group	Diagnosis	No. of cases	Mean Period for Monitor
Group 1	Osteosarcoma	20	17.8 months
Group 2	Benign bone tumor	34	27.6 months
Group 3	2nd Bone Metastasis	26	18.8 months

2.1.1 Monitering the plasma bone specific ALP

2.1.1.1 Osteosarcoma group

At the time of admission, history of the patien, especially the time for developing the symptom, and physical examaination were recorded. Biochemical investigation included the blood routine, plasma calsium, phosphate, and alkaline phosphatase isoenzyme was also recorded. Radiological investigation include the CXR, XR of two dimension of the tumour, bone scan, MRI etc was used to excluded any metastasis of the disease. Diagnosis was confirmed only by the

histopathological result obtained at the time of biopsy, or during operation.

Plasma alkaline phosphatase isoenzyme was measured at the time of diagnosis, in each course of pre-operational and post-operational chemotherapy, the day before and after operational removal of tumour or amputation, and during every follow-up clinic. In the period of follow-up, CXR was taken every 3 months or at the time when clinical sign or symptome suggest recurrent of disease.

2.1.1.2 Benign bone tumour group

Similar to the osteosarcoma group, history, physical sign, biochemical and radiological investigation were recorded at the time of admision. Diagnosis was base on the histopathological result. Plasma alkaline phosphatase isoenzyme was measured at the time of diagnosis, and each time of follow-up clinics.

2.1.1.3 Metastasis group

Clinical history, physical sign, and investigation, including the alkaline phosphatase isoenzyme, were recorded during the time of referal from the Oncology Department. The diagnosis of primary malignant tumour and the result of bone metastasis was made only by the histopathological report.

2.1.2 Collection of plasma samples preserve of tumor tissue

Patients arm was placed in extend position. Cephalic vein or Median Cubital vein was located. Tunaquited the arm and sterilized the puncture site with alcohol swab *(BRAND)*. Venepunture was performed and 5 ml venous blood was aspirated with a 10 ml sterile syringe *(TERUMO)*. Unfastened the tunaquit and withdrawed the syringe. Removed the neddle and immediately transferred the

blood into a 10 ml heparinized tube containing beads coated with ammonium heparine *(SARSTEDT)* and mixed the tube gently for about 30 times. Plasma sample was obtained by centrifugation at 1,000 xg for 10 minutes and aliquoted into 3 Eppendorfs and stored at -70°C.

In the Excision and Allograft operation, the entire tumor was excised with clear margine. The tumor tissue was splitted into 2 half with electric saw or bone cutter. One half was send to the Pathological Department for histo-pathological diagnosis while the second half was kept for biochemical assay. The halvested tumor tissue was washed twice gently with normal saline, each for 2 minutes, to ensure that all the blood on the surface of the tumor was cleaned out. Then, the tumor was cutted into 10 equal parts and wraped inside double plastic bag with lable of the location and stored at -70°C.

Photo 2-1 Osteosarcoma tissue halvested during the Excision and Allograft operation and splited into 2 half. One for histopathological diagnosis and the other for biochemical assay.

blood into a 10 ml heparinized tube containing beads coated with ammonium heparine *(SARSTEDT)* and mixed the tube gently for about 30 times. Plasma sample was obtained by centrifugation at 1,000 xg for 10 minutes and aliquoted into 3 Eppendorfs and stored at -70°C.

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Photo 2-1 Osteosarcoma tissue halvested during the Excision and Allograft operation and splited into 2 half. One for histopathological diagnosis and the other for biochemical assay.

2.2 QUANTITATIVE ANALYSIS OF THE PLASMA AND TISSUE BONE SPECIFIC ALKALINE PHOSPHATASE

The method described by Rosalki and Foo in 1984 was adopted for the assay of bone-specific ALP. The original method required two-step incubation with a pre-treatment ot Triton X-100 provided that serum sample demonstrated the presence of biliary ALP. The pre-incubation was time consuming and could compricate the calculation of bone-specific ALP activity. The original author subsequently modified their method in 1986, so that only one step incubation would be necessary for the precipitation of bone-specific ALP. The modified method was adopted in the assessment of bone-specific ALP in the plasma samples and tumour tissue extracts.

2.2.1 Extraction of tissue ALP

2.2.1.1. Reagent

Borate Buffer (0.1M, pH 7.03)

6.183g boric acid (H_3BO_3 : MW 61.83) was dissolved in approximately 950 ml distilled water (pH = 5.5). The acid was titrated to pH 7.03 with 0.2 NaOH at 18°C - 20°C. The solution was diluted to 1 L with distilled water.

2.2.1.2. Homogenization of the bone tissue

The bone tissue that freezed at -70°C was brought to room temperature and washed with ice-cooled 0.1 M sodium borate buffer (pH 7.03). The bone tissue was crushed into small pieces with the bone cutter, then, homogenized with liquid nitrogen in stainless steel homogenizer (Photo 2-2) cooled at -70°C overnight before homogenization. The homogenates were transferred to a pre-

weighted 1.5 ml Eppendorf tube. The wet weight of the sample was recorded. Then the Eppendorf tube was covered with parafilm paper on which some small holes were pinned with a needle. The sample was subjected to lyophilization for 24 hours by lyophilizer *(LABCONCO)*. After lyophilization, the dry weights of the sample was recorded. This sample was stored at -70°C or subjected to ALP extraction immediately.

2.2.1.3. Extraction of ALP

The sample was transferred to pre-weighed 12 mm x 75 mm opaque plastic tube and the dry weight of the sample transferred was recorded. To each sample, 2 ml 0.1 M sodium borate buffer (pH 7.03) was added and the ALP was extracted with constant mixing by inversion mixer for 24 hours at 4°C. The mixture was then centrifuged at 3,500 x g for 20 minutes at 4°C *(Beckman AccuSpin FR)*. One hundred folds aqueous solution of the supernatant was prepared and the diluted supernatant was taken for routine ALP assays.

Photo 2-2 Stainless steel homogenizer used to crush and homogenized the tumour tissue

2.2.2 Assay for Bone-specific ALP

2.2.2.1. Reagents

Wheat Germ Lectin / Triton X-100 Mixture

Lectin of wheat germ from *Triticum vulgaris* was purchased from *SIGMA* & Triton X-100 from *Scintran BDH*. A solution containing 0.0275 g lectin and 0.22 g Triton X-100 in 5.5 ml distilled water. Complete dissolution was achieved by vortexing the solution at least 30 minutes.

p-Nitrophenylphosphate Substrate Solution for Autoanalysor

(AMP Buffer Solution, *American Monitor Cooperation*)

2.2.2.2. Procedures

The plasma sample or the bone tissue extract stored at -70°C was thawed & brought to room temperature by 37°C water bath. Precipitation of bone-specific ALP was achieved by mixing 50 µL plasma sample or diluted bone tissue extract with equal volume of wheat germ lectin/Triton X-100 solution. The mixture was incubated at 37°C in shacking water bath for 30 minutes. After incubation, the mixture was centrifuged at 2,000 x g for 10 minutes. The supernatant was taken for the estimation of non-bone ALP activity.

Alkaline phosphatase kinetic assays were assessed by the hydrolysis of *p*-nitrophenylphosphate at alkaline medium performed by *COBAS BIO* autoanalysor *(ROCHE Diagnostica)*. The liberated *p*-nitrophenylate shows maximum absorption at 405 nm. The rate of the increase in yellow color is directly proportional to the alkaline phosphatase activity in the sample.

50 µL sample was pipetted to the tube for COBAS BIO autoanalysor. Photo 2-2. After the reagent chamber was filled with *p*-nitrophenylphosphate substrate solution and the cuvette ring was loaded, the machine was set to 'SINGLE RUN' and with the Test Number set to "25". We can start to measure the value of each sample tube. The Test "25" was previously set for measurment of the alkaline phosphatase with the followering parameters :

1.	UNITS	U/L
2.	CALCULATION FACTOR	2258
3.	STANDARD 1 CONC	0
4.	STANDARD 2 CONC	0
5.	STANDARD 3 CONC	0
6.	LITMIT	0
7.	TEMPERATURE [DEG. C]	37.0
8.	TYPE OF ANALYSIS	2
9.	WAVELENGTH [NM]	405
10.	SAMPLE VOLUME [UL]	6
11.	DILUTENT VOLUME [NM]	50
12.	REAGENT VOLUME	250
13.	INCUBATION TIME [SEC]	0
14.	START REAGENT VOLUME [UL]	0
15.	TIME OF FIRST READING [SEC]	110
16.	TIME INTERVAL [SEC]	10
17.	NUMBER OF READINGS	15
18.	BLANKING MODE	1
19.	PRINT OUT MODE	1

For each sample, pair assays were performed, one for the total ALP in plasma or diluted bone tissue extract, and the other for the non-bone ALP in the supernatant of that sample after treated with lectin solution. Bone-specific ALP activity was calculated from the difference between the two values, as shown below:

Bone-specific ALP (U/L) = Total ALP (U/L) - 2 x Non-bone ALP (U/L)

Control sera from human (Total ALP activity = 150.74 ± 4.08 U/L) and rabbit serum (Total ALP activity = 104.51 ± 2.40 U/L) were included in each run of 22 assays.

2.3 QUALITATIVE MEASUREMENT OF ALP ISOENZYME

(Analytical Electrofocusing in Carrier Ampholyte pH)

2.3.1 Equipment required

Instruments and accessories

Multiphor ll electrophoresis unit with MultiDrive XL 3.5 kV power supply
MultiTemp II thermostatic circulator (220 v)
Surface pH electrode with support arm and cooling jacket
Humidity chamber

Agarose gels

Agarose IEF
GelBond PGA film
IEF electrode strips

Consumable Reagents

Ampholine, pH 3.5-5.0
Ampholine, preblended, pH 3.5-9.5

All the above apparatus manufactured by*Pharmacia Biotechnology AB Uppsala, Sweden.*

2.2.2 Practical procedure

2.3.3.1 Gel casting

Reagents

Agarose	0.25 mg
Sorbital	2.50 mg
Ampholyte with pH 3.5-9.5	2.00 ml
Milliqute distilled water	23.00 ml

Procedures

1. Cut the GelBond PAG film in tho the size 10 cm x 13 cm. Pour about 2 mL of 75% ethanol onto the hydrophilic side of the Gelbond film and rubbing with Kleenic paper, this reduce the adhersive force of the GelBond PAG film.

2. Level the leveling plateform by mount the levelling feet by turning the gel casting table upside down and screwing one foot into each corner. Pour 0.4 ml of distilled water on it and this is used to adhere the GelBond PAG film firmly onto the plateform.

3. Lay a GelBond PAGfilm over it with the hydrophilic side up. Preheat the film by blowing hot air from a electric hair dryer, which is clipped by a stand 10 cm above, for about 5 minutes.

4. Prepare the appropriate volume of gel solution (25 ml).

Combine 0.25 gm agarose, 2.5 gm sorbital and 23 ml distilled water in an Erlenmeyer flask. Place the flask in a cold water bath and heated to boiling while stiriling. When the agarose is disolved completely, decrease the temperature to 75^{o}C. Add 2 ml of the Ampholine with pH 3.3 - 9.5 and mixed throughly.

5. Pour the gel solution gently onto the GelBond PAG film. Tilting of the leveling plateform is allowed to spread the gel solution evently on the film. Check that there are no air bubbles in the gel and make sure the plateform is leveled.

6. Wait for 2 minutes to allow the gel cool and forming a 1 mm thick agarose gel for supporting media.

2.3.3.2 Sample preparation and application

1. Applied 3 μLthe plasma sample or the tissue extract or the molecular weight marker with a 10 μL pipette. The plasma sample was freshed, undialysed and undiluted.

2. The sample should be applied along the axis of the elctrophoresing direction. And recorded down the lable of the different bands.

2.3.3.3 Electrofocusing

Reagent

Electrode solutions (pH range 3.5-9.5)

Anode

0.025 M Aspartic acid - 0.33 g Aspartic acid (MW:132)

0.025 M Glutamic acid - 0.37 g Glutamic acid (MW:146)

dissolve in distilled water to a total volume of 100 mL.

Cathode

2.0 M Ethylenediamine - 13.2 mL Ethylenediamine (MW:60)

0.025 M Arginine - 0.44 g Arginine (MW:174)

0.025 M Lysine - 0.40 g Lysine (MW:164)

make up to 100 mL with distilled water. Stir until clear.

Procedures

1. Set MultiTemp II thermostatic circulator to 10°C, and switch it on 15 minutes before beginning the experiment.

2. Apply the agarose gel to the cooling plate. Place it in the center of the cooling plate.

3. Apply the electrode strips wetted with the appropriateelectrode solutions.
4. Apply the samples as discribed on the above section.

Running conditions for 1mm thick agarose gel in pH range 3.5-9.5

Voltage (V)	500 V	[MAX]
Current (mA)	50	[MAX]
Power (W)	15	[MAX]
Time (min)	10 Hr	[MAX]

2.3.3.4 Western blotting of the protein

One of the major recent advances in the analysis of protein after polyacrylamide or agarose gel electrophoresis has been the development of techniques for the transfer of the seperated protein from the gel to a thin support matrix, most commonly a nitrocellulose membrane, to which they bind and immobilized. This transfer procedure is referred to as a blotting. Under ideal condition, over 90% of the protein can be transfered.

Reagent

1) Transfer Buffer (pH8.3)

Tris-Base	3.0275 g/L
Glycine	14.4885 g/L
Methanol (20%)	200 ml/L

2) Assay Buffer (pH 7.2)

Tris-HCl	7.88 g/L
Sodium Chloride	8.775 g/L

3) Blotting apperatus kit

4) Absorber, the filter paper and paper towers.

5) Hybon C membrane

6) Plastic Warp sheet

Procedures

1. Set up the blotting apperatus as the figure 2-1 below.

2. Marked the surface of the Hybon C membrane that contact to the agarose gel which containing the seperated protein. And mark the position of sample application.

3. Allow blotting for 1 hour

4. Wash twice the transfered membrane with assay bufer, each 10 minutes.

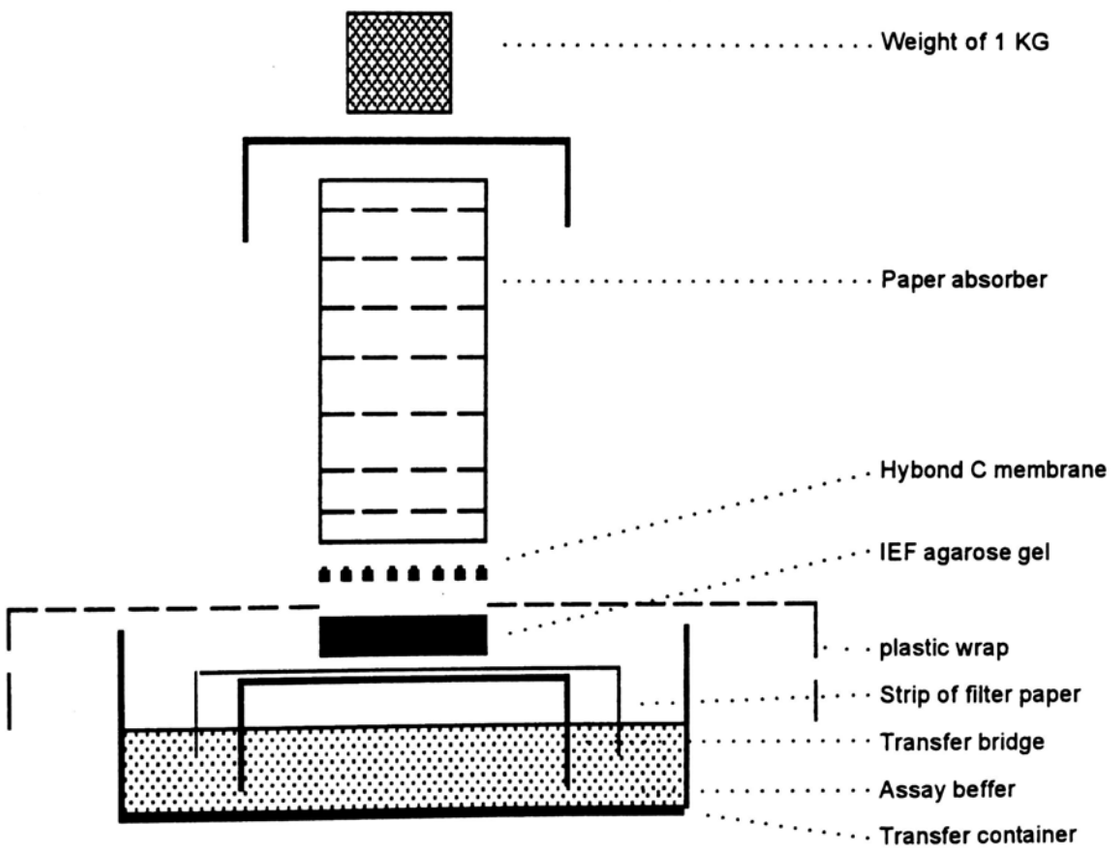


Figure2-1 Set up of the Western blotting apperatus

2.3.3.5 Detection methods

i) Coomassie Blue R 250 stain

The use of Coomasie Blue R 250 stain is for the protein staining. Therefore, not only the alkaline phosphatase but also all the protein in the plasma or tissue extract was stained by this stain. Moreover, the molecular weight marker was also stained by this Coomasie Blue R 250 stain.

Reagent

1) Fixing solution

350 mL Methanol

130 g Trichloroacetic acid

35 g Sulphosalicylic acid

make up to 1 Liter with distilled water and stir until completely dissolved.

2) Preserving solution

50 mL Glycerol (87%)

175 mL Ethanol (95%)

50 mL Acetic acid

make up to 500 mL with distilled water and mix thoroughly.

3) Staining solution

1.5 g Coomassie Blue R 250

105 mL Ethanol (95%)

30 mL Acetic acid

make up to 300 mL with distilled water and stir.

4) Destaining solution

350 mL Ethanol (95%)

100 mL Acetic acid

make up to 1 Liter with distilled water and mix thoroughly.

Procedure

1. Immediately after focusing remove the electrode strips
2. Place the gel in fixing solution (1) for 5 minutes.
3. Place the gel in 100 mL destaining solution (4) for 5 minutes.
4. Place the gel in staining solution (3) at room temperature for 5 minutes.
5. Transfer the gel to destaining solution (4) for two 10 minute washes, or until the background is clear.
6. Preserve the gel by leaving it in preserving solution (2) for 5 minutes.
7. Dry the gel using a stream of hot air by a electric hair dryer, which is clipped 10 cm above.

ii) Indoxy phosphate-formazan stain

Reagent

Staining solution together with the substrate of the alkaline phosphates

0.1 M Tris Buffer solution	50 ml
$MgSO_4$	5 mM
MTT [3-(4,5-dimethylthiazole-2)-2,5-diphenyltetrazolium bromide]	100 µg/ml

Procedure

1. Put the transfered membrane into a tube container. Pour the substrate and staining into the container. The substrate should be freshly prepared.

2. Incubate at 37°C for 16 hours.

3. Under this condition, the reaction is carried out in the dark. Therefore, tin foil paper is used to warp the container tube during the whole process.

4. Enzymatic activity of the alkaline phosphatse isoenzyme appears as the blue bands.

5. The molecular weight of the specific band was calculated according to the position of the different positions of the molecular weight marker stained by the Coomassie Blue R 250 stain.

iii) Imunological antibody-antigen stain

Reagent

1) 200 ml 2 % skin milk

2) First antibody

Peroxidase conjugated anti-mouse IGG 1:1000

3) Second antibody

Peroxidase conjugated sheep-anti-mouse IGG 1:500

4) ECL Detecting agent (Amersham Int. plc, Amersham UK)

Procedures

1) Place the transferred membrane into 2 % skin milk for one hour. All the enzyme on the membrane was binded with the skin milk protein.

2) Treat the membrane with first antibody for 5 minutes. Only the ALP isoenzyme will be released from the skin milk protein and bine to the first anti-body.

3) Wash with Assay buffer twice, each for 5 minutes. Then, add the second anti-body. Mix thouroughly with the second antibody for 5 minutes.

4) Wash again with Assay buffer twice, each for 10 minutes.

5) Add the detecting agent and let the reaction run for 1 minute. The product after the reaction is flurorescent. Take out the membrane and wrap it with a transpeerent plastic wrap.

6) Put an X-ray film on the membrane inside a X-ray box. The exposing time for the plasma sample is 10 seconds, and 3 minutes for the sample of tissue extract.

7) Develope the X-ray film, bands of the plasma and tissue ALP isoenzyme could be observed on the X-ray film.

2.4 METHOD OF STATISTICAL ANALYSIS

The statistical results were analyzed by **SPSS for Window, Release 5.0** ***(© SPSS Inc., 1989-1992.).*** Including the Mean and Standard deviation, Wilcoxon Matched-Pair Signed-Ranks Test, Oneway ANOVA and Duncan's Mutiple Ranged Test was used.

Due to the fact that normal plasma ALP activity varies a lot with in different age group. We use Match- Paired Test to test the significance of the mean of the individual pair in each patients according to the different age group. Moreover, since the number of sample is so small that the Student's T-Test cannot be used. The Non-parametric Test is the test of choice. Therefore, in the present study, the Wilcoxon Matched-Pair Signed-Ranks Test is used. Oneway ANOVA is the test used to compare the significance of the mean of more than two groups, it showed the significant between the groups but the details of p value between each pair of means is proved by the Duncan's Mutiple Ranged Test.

Chapter 3
Results

3.1 QUANTITATIVE MEASUREMENT OF PLASMA AND TISSUE BONE SPECIFIC ALKALINE PHOSPHATASE

3.1.1 General Information of the patients monitoring

In the period of October 1991 to April 1993, 20 patients with osteosarcoma; 34 patients with benign bone tumour and 26 patients with malignant tumour that metastased to the skeletal system were recorded when they were admitted or referred to the Department of Orthopaedics and Traumatology, Prince of Wales Hospital. They were divided into 3 groups, namely the Osteosarcoma group, the Benign group and the Metastasis group respectively.

In the Group 1, the patients with osteosarcoma, we have 4 patients with the age equal 12 years, and 16 patients above 16 year of age. The average age is 20.25 year. The male patient is predominated with the male to female ratio 16 : 4. Histologically, there are 16 cases of osteoblastic osteosarcoma, 3 cases of chondroblastic osteosarcoma and 1 case of telengietic osteosarcoma. Radiologically, the tumor was classified as osteosclerotic (8 cases) when radiodense neoplastic bone dominated, osteolytic (5 cases) when radiolucency predominated and mixed (7 cases) when they were equal portion of each radiographic aspect. Most of the patients received 4 courses of pre-operational and post-operational chemotherapy. One case (SC) developed acute hepatitis A virus infection (HAV) and another case (PTM) refuse the pre-operational chemotherapy. Most of the patients under went Excision and Allograft operative treatment.

In the period of follow-up, 11 patients had recurrence of the osteosarcoma. The first metastasis site were located in lung in 5 cases and 2 cases in spine, on the other hand, 4 cases developed local recurrence in the operative site. The average recurrent time was 40.36 weeks. The 9 other patients remain disease

free after the operation, with the mean follow-up period of 102.11 weeks. Therefore, in the present study, the group 1 patients (osteosarcoma) were subdivided into 2 sub-groups, namely, the recurred osteosarcoma group (Group 1R) and the non-recurred osteosarcoma group (Group 1N). The general information of the group 1 patient was summarized in the following table.

Table 3.1 General information of the osteosarcoma patient group.

Group	Name	Age	Date Dx.	Diagnosis	X-ray	POC	Operaion	PSC	2nd. Met.
Gp 1R	AKM	18/M	15-Jul-92	C-B-S L illius	L+S	4	17-Feb-93 (E)	2	13-Aug-93
	CLY	14/M	20-Jan-92	T-Ost. R pelvic	S	4	10-Jun-92 (E)	0	9-Nov-92
	KCY	10/M	29-Aug-91	C-B-S L tibia	S	4	3-Jan-92	4	26-May-92
	LWS	13/M	20-Nov-92	Ost. R tibia	L+S	4	8-Mar-93 (E+A)	3	15-Feb-93
	LMC	32/M	21-Sep-92	C-B-S pelvic	L	2	9-Nov-92 (E+R)	4	18-Jan-94
	PTM	14/M	18-Feb-93	R tibial ost.	L	0	26-Feb-93	4	27-Apr-94
	SKW	11/M	13-Sep-91	Ost. L femur	S	2	24-Nov-91	4	30-Apr-94
	SC	24/M	23-Jun-92	Ost. R tibia	S	0	15-Jul-92 (E+A+N)	0	13-Jul-93
	TYC	38/M	24-Apr-92	Ost. L femur	S	2	3-Jul-92	0	5-Sep-92
	TCY	18/F	11-Sep-91	Ost. R tibia	L	4	27-May-91 (E+A)	4	13-Nov-92
	WCK	12/M	11-Mar-92	Ost. R tibia	S	1	7-Apr-92	4	5-Jan-93
Gp 1N	CHL	15/M	8-Sep-92	Ost. R femur	L+S	4	23-Oct-92 (E+A)	4	
	CY	27/F	11-Sep-91	Ost. R tibia	L+S	4	12-Jan-92 (E+A)	4	
	KSP	18/F	23-Sep-91	Ost. R humerus	L	4	26-Mar-1992 (E+A)	4	
	LFH	39/M	22-Oct-92	Ost. R knee	L	4	12-Feb-93 (E+A)	3	
	LKH	14/M	8-Sep-92	Ost. R humerus	L+S	4	5-Mar-93 (E + A)	3	
	LW	36/F	2-Sep.-91	Ost. L tibia	L+S	4	25-Jan-92	4	
	MSK	20/M	1-Sep.-91	Ost. L tibia	S	4	3-Apr-1992 (E+A)	3	
	TCC	20/M	1-Oct-92	Ost. L tibia	L+S	4	17-Jul-93	?	
	TCW	12/M	16-Dec-91	Ost. L pelvic	S	4	12-May-93	4	

C-B-S : Condroblastic osteosarcoma
Ost. : Osteoblastic osteosarcoma
T-Ost. : Telengietic osteosarcoma
L + S : Radiological mixed tumor
L : Radiological osteolytic tumor
S : Radiological osteosclerotic tumor

POC : Preoperational chemotheraphy
PSC : Postoperational chemotheraphy
Meta. :Date of clinically detectd metastasis

In the group 2, the patients with the benign bone tumor, we have 34 patients aged from 23 year to 55 year with average 32.8 years. 23 of them had disease of Giant Cell Tumor, 5 developed Echondroma, 3 cases of Osteoma and 3 cases of Chondroma. The mean follow-up time was 27.6 months.

In the group 3, the patients with malignant tumour that migrated to the skeletal system, they were older patients, with the average 63.7 years of age. The primary tumor site was from breast in 9 cases, 8 cases from lung, 4 cases from cervix and 3 cases from intestine. The mean follow-up period for this group of patients was 18.8 months.

3.1.2 Pretreatment evaluation

At the presentation, both the plasma total and bone specific ALP level was elevated in all the 20 osteosarcoma patient. The mean value of the total alkaline phosphatase TALP level was 343.90 IU/L and with S.D. 180.64 IU/L, while the mean value of the bone specific alkaline phosphatase BALP in plasma was 302.65 IU/L with S.D. 180.19 IU/L. The mean value of the NBALP in plasma was 41.25 IU/L with S.D. 11.80 IU/L. When Wilicoxon Matched-Pairs Signed-Ranks Test was performed, both the plasma TALP and BALP level were **significantly higher** than the normal value, $p < 0.001$. However, the non-bone portion of the alkaline phosphatase NBALP remained in the normal range.

The mean value of the plasma TALP and BALP activity in the patients with begnine bone tumor, the group 2 patients, was 79.68 IU/L with S.D. 22.69 IU/L, and 31.29 IU/L with S.D. 10.27 IU/L, respectively. When Wilicoxon Matched-Pairs Signed-Ranks Test was performed, both the plasma TALP and BALP level were **NOT significantly** higher than the normal value, $p > 0.05$.

The mean value of the plasma TALP and BALP activity in the group 3, the patients with malignant tumour that migrated to the skeletal system, was

114.58 IU/L with S.D. 172.42 IU/L, and 34.15 IU/L with S.D. 8.17 IU/L, respectively. When Wilicoxon Matched-Pairs Signed-Ranks Test was performed, both the plasma TALP and BALP level were **NOT significantly higher** than the normal value, $p > 0.05$.

When comparison of the plasma BALP and TALP level between the group 2 and group 3 patient was performed, the Wilicoxon Matched-Pairs Signed-Ranks Test showed that there was **no significantly difference** between the two groups of patients. The p value of the plasma TALP between the 2 groups is 0.545 while the p value of the plasma BALP between the 2 groups is 0.079. Although both the p value is larger than 0.05, the plasma BALP significance is still better than that of the plasma TALP.

By using the Oneway ANOVA, it was shown that there was a significant difference of the plasma BALP level in the osteosarcoma patient group (group 1) amongst the normal control, group of patients having begnine bone tumor (group 2), and that of the group of patients with primary malignant tumor metastasis to the skeletal system (group 3). $F(3, 106) = 64.0544$, $P < 0.0001$. The Duncan Multiple Range Test was applied to asses the groups that contributed to the overall significance. The results are outlined in table 3-2 below, where 'X' was marked between the specific 2 groups that have the significant difference at the level $p < 0.05$.

Table 3-2 Duncan test showing the plasma BALP level of the corresponding groups of patients having a significant difference, as indicated by 'X'.

Mean	Group of patients	Group 1	Group 2	Group 3	Normal
305.15	Group 1 (osteosarcoma)		X	X	X
34.2941	Group 2 (Begnine tumor)	X			
34.1538	Group 3 (Bone metastasis)	X			
56.0000	Normal BALP value	X			

Moreover, in the group 1 patients, when the value of BALP was divided by that of the TALP, we found that the bone portion of the plasma alkaline phosphatase enzyme was above 85%, with mean value 85.36% S.D. 7.50, it was significantly higher than the that of the normal subjects with portion of BALP in plasma 40%, $p < 0.001$. On the other hand, the bone portion of the group 2 and group 3 was 39.39% with S.D. 5.91 and 32.33% with S.D. 10.07, respectively. The percentage was similar to that of the normal subjects. The detail result was recorded in table 3-3.

Table 3-3 Mean and S.D. of the plasma ALP isoenzyme and the percentage of the BALP in different groups of patients.

	Group 1			Group 2			Group 3		
	TALP	*BALP*	*%*	*TALP*	*BALP*	*%*	*TALP*	*BALP*	*%*
	915	873	95.41	75	42	56.00	96	39	40.625
	172	136	79.07	90	36	40.00	98	43	43.88
	282	241	85.46	74	27	36.49	119	32	26.89
	342	316	92.40	62	21	33.87	74	22	29.73
	445	390	87.64	47	21	44.68	81	26	32.10
	184	156	84.78	89	34	38.20	100	27	27.00
	151	108	71.52	101	38	37.62	48	21	43.75
	322	285	88.51	128	54	42.19	66	27	40.91
	241	205	85.06	63	26	41.27	51	20	39.22
	177	120	67.80	63	27	42.86	119	31	26.05
	517	449	86.85	106	49	46.23	106	33	31.13
	303	250	82.51	120	52	43.33	103	37	35.92
	344	310	90.12	87	34	39.08	68	29	42.65
	258	203	78.68	113	47	41.59	169	35	20.71
	504	480	95.24	86	31	36.05	193	35	18.13
	189	145	76.72	44	20	45.45	105	31	29.52
	415	381	91.81	89	28	31.46	103	42	40.78
	238	212	89.08	60	26	43.33	101	33	32.67
	327	280	85.63	78	23	29.49	83	30	36.14
	552	513	92.93	84	27	32.14	105	39	37.14
				89	34	38.20	126	42	33.33
				46	22	47.83	914	57	6.24
				73	29	39.73	429	42	9.79
				98	41	41.84	83	38	45.78
				103	35	33.98	108	41	37.96
				73	20	27.40	111	36	32.43
				61	27	44.26			
				100	46	46.00			
				107	41	38.32			
				68	25	36.76			
				78	23	29.49			
				40	16	40.00			
				69	25	36.23			
				45	17	37.78			
Mean	343.90	302.65	85.36	79.68	31.29	39.39	144.58	34.15	32.33
S.D.	180.64	180.19	7.50	22.69	10.27	5.91	172.42	8.17	10.07

Futhermore, the high S.D. value of the TALP in the group 3 patients, 172.42 IU/L which is even higher than that of the mean value. This showed that the plasma TALP measurement was not as good as that of the plasma BALP measurement.

The mean value and S.D. of the three groups of patients were summarised in the following figure 3-1 and the details of the values was in the table 3-4.

Figure 3-1 Mean and S.D. of the plasma ALP level in the three groups of patients. Group 1, the group of patients with osteosarcoma, group 2, The group of patients with begnine bone tumor and group 3, the group of patients with primary malignant tumor metastasis to skeletal system. TALP is the total plasma alkaline phosphatase, BALP is the plasma bone specific alkaline phosphatase and the NBALP represent the plasma non-bone portion alkaline phosphatase.

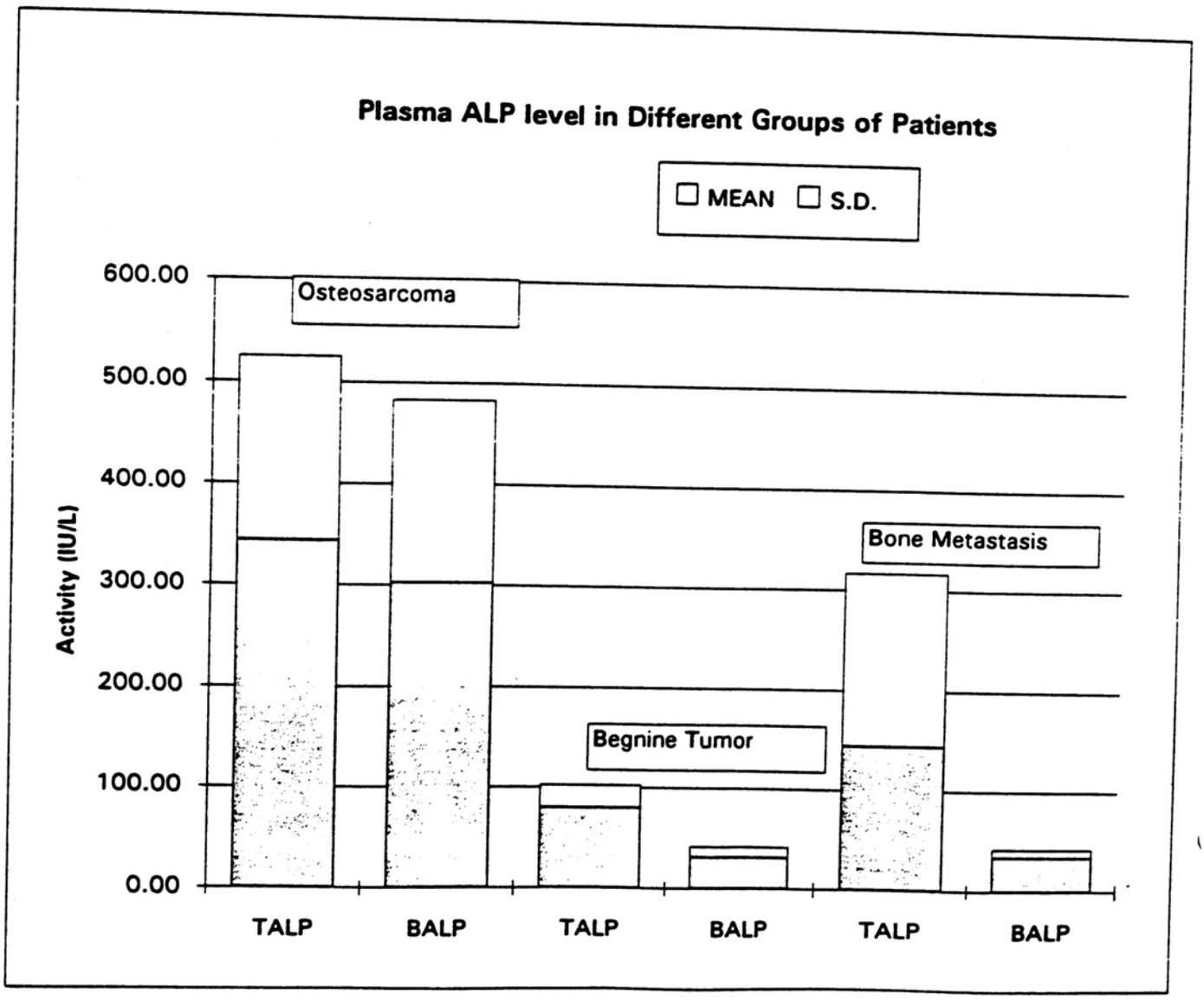


Table 3-4 Mean and S.D. of the plasma ALP level in the three groups of patients. Group 1, the group of patients with osteosarcoma, group 2, The group of patients with begnine bone tumor and group 3, the group of patients with primary malignant tumor metastasis to skeletal system. TALP is the total plasma alkaline phosphatase, BALP is the plasma bone specific alkaline phosphatase and the NBALP represent the plasma non-bone portiion alkaline phosphatase.

GROUP 1	Diagnosis			GROUP 2	Diagnosis			GROUP 3	Diagnosis		
	TALP	BALP	NBALP		TALP	BALP	NBALP		TALP	BALP	NBALP
C-B-S L ilius	915	873	42	GCT	75	42	33	Ca Breast	96	39	57
Ost. R tibia	172	136	36	GCT	90	36	54	Ca Breast	98	43	55
Ost. R femur	282	241	41	GCT	74	27	47	Ca Breast	119	32	87
T-Ost. R pelvic	342	316	26	GCT	62	21	41	Ca Breast	74	22	52
C-B-S L tibia	445	390	55	GCT	47	21	26	Ca Breast	81	26	55
Ost. R humerus	184	156	28	GCT	89	34	55	Ca Breast	100	27	73
Ost. R knee	151	108	43	GCT	101	38	63	Ca Breast	48	21	27
Ost. R humerus	322	285	37	GCT	128	54	74	Ca Breast	66	27	39
C-B-S pelvic	241	205	36	GCT	63	26	37	Ca Breast	51	20	31
Ost. L tibia	177	120	57	GCT	63	27	36	Ca Lung	119	31	88
Ost. R tibia	517	449	68	GCT	106	49	57	Ca Lung	106	33	73
Ost. L tibia	303	250	53	GCT	120	52	68	Ca Lung	103	37	66
R tibial ost.	344	310	34	GCT	87	34	53	Ca Lung	68	29	39
Ost. R tibia	258	203	55	GCT	113	47	66	Ca Lung	169	35	134
Ost. L femur	504	480	24	GCT	86	31	55	Ca Lung	193	35	158
Ost. L tibia	189	145	44	GCT	44	20	24	Ca Lung	105	31	74
Ost. L pelvic	415	381	34	GCT	89	28	61	Ca Lung	103	42	61
Ost. R tibia	238	212	26	GCT	60	26	34	Ca Cervix	101	33	68
Ost. L femur	327	280	47	GCT	78	23	55	Ca Cervix	83	30	53
Ost. R tibia	552	513	39	GCT	84	27	57	Ca Cervix	105	39	66
				GCT	89	34	55	Ca Cervix	126	42	84
				GCT	46	22	24	Ca Liver	914	57	857
				GCT	73	29	44	Ca Liver	429	42	387
				Echondroma	98	41	57	L Intestine	83	38	45
				Echondroma	103	35	68	L Intestine	108	41	67
				Echondroma	73	20	53	L Intestine	111	36	75
				Echondroma	61	27	34				
				Echondroma	100	46	54				
				Osteoma	107	41	66				
				Osteoma	68	25	43				
				Osteoma	78	23	55				
				Chondroma	40	16	24				
				Chondroma	69	25	44				
				Chondroma	45	17	28				
MEAN	343.90	302.65	41.25	MEAN	79.69	31.29	48.38	MEAN	144.58	34.15	110.42
S.D.	180.64	180.19	11.80	S.D.	22.69	10.27	14.27	S.D.	172.42	8.17	167.02

3.1.3 Correlation between the pretreatment plasma ALP levels and prognosis in the osteosarcoma patient group

The mean value and S.D. of the group 1 osteosarcoma patients were summarized in the following figure 3-2, we subdivided the group into 2 subgroups, the recurrent group (group 1R) and the nonrecurrent group (group 1N), while the details of the values was recorded in the table 3-5.

Mean and S.D. of plasma ALP isoenzyme in group 1 patients

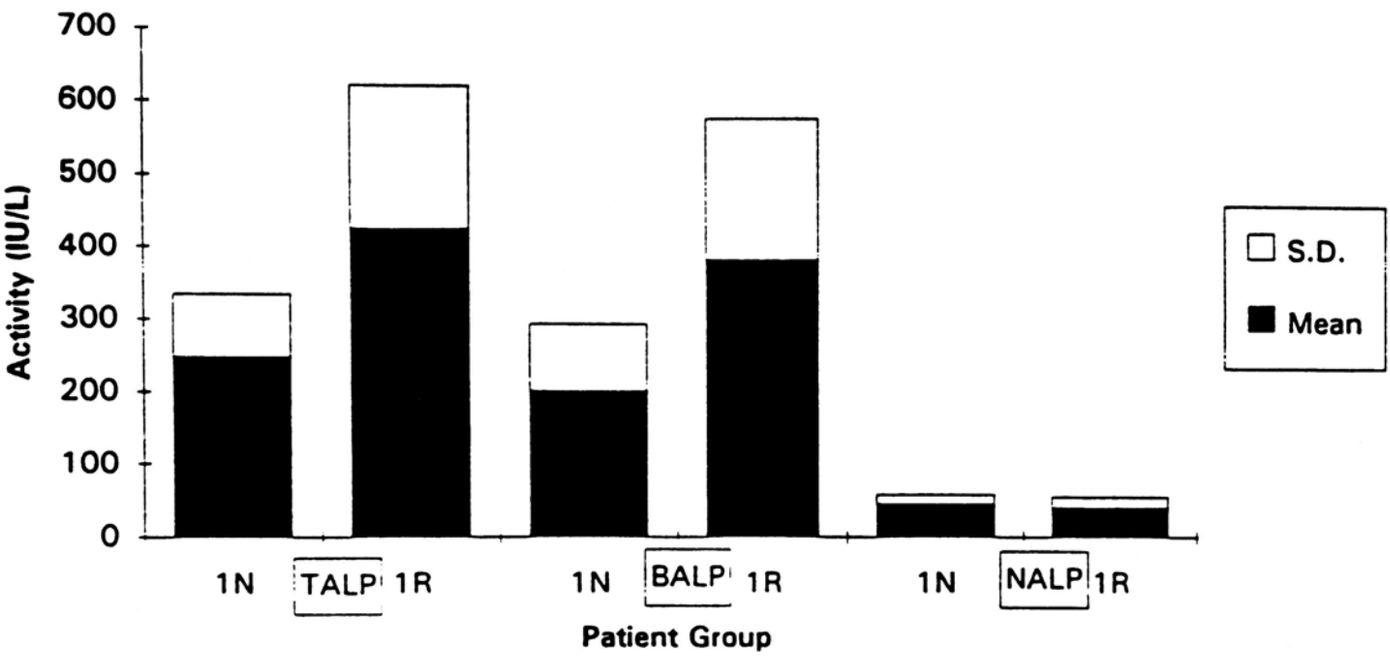


Figure 3-2 Mean and S.D. of the plasma TALP and BALP level in the 2 subgroup 1N and 1 R patients at the time of diagnosis.

At the time of diagnosis, the mean value of the plasma TALP level in the group 1R (osteosarcoma patients with recurrent disease) was 425.73 IU/L, S.D. 197.76 IU/L, while the mean value of the plasma BALP was 248.33 IU/L with S.D. 92.22 IU/L. When Wilicoxon Matched-Pairs Signed-Ranks Test was performed, both the plasma TALP and BALP level were significantly higher than the corresponding value of the group 1N (osteosarcoma patients without recurrence), with Z value = -2.3102, 2-Tailed P = 0.0209 which means $p < 0.05$. However, the non-bone portion of the alkaline phosphatase (NBALP) has **no significantly** difference between the 2 subgroups 1N and 1R.

Table 3-5 Details of the results of the Mean and S.D. of the of plasma TALP, BALP and NBALP level in the osteosarcoma patients with (group 1R) and without (group 1N) recurrent of disease. TALP is the total plasma alkaline phosphatase. BALP is the plasma bone specific alkaline phosphatase and the NBALP represent the plasma non-bone portion alkaline phosphatase.

Name	F.U. (Weeks)	Diagnosis			Pre-operation			Post-operation			During Follow-up		
		TALP	BALP	NBALP	TALP	BALP	NBALP	TALP	BALP	NBALP	TALP	BALP	NBALP
CHL	120	282	241	41	324	286	38	140	107	33	104	61	43
CY	119	172	136	36	114	82	32	76	35	41	64	27	37
KSP	117	204	156	40	123	69	54	75	28	47	63	31	32
LFH	66	171	108	73	143	74	69	107	36	71	69	22	47
LKH	71	322	285	37	139	110	29	109	73	36	68	36	32
LW	120	177	120	57	96	50	46	80	36	44	72	26	46
MSK	120	303	250	53	155	98	57	133	90	43	140	85	55
TCC	68	189	145	44	134	85	49	125	83	42	87	43	44
TCW	118	415	381	34	135	98	37	183	135	48	76	42	34
MEAN	102.11	248.33	202.44	46.11	151.44	105.78	45.67	114.22	69.22	45.00	82.56	41.44	41.11
S.D.	25.38	86.39	92.22	12.69	66.96	69.90	13.00	35.56	37.86	10.86	25.14	20.14	7.88

Name	Recurre (Weeks)	Diagnosis			Pre-operation			Post-operation			At Recurrence		
		TALP	BALP	NBALP	TALP	BALP	NBALP	TALP	BALP	NBALP	TALP	BALP	NBALP
AKM	52	915	873	42	254	215	39	157	104	53	733	692	41
CLY	38	342	316	26	162	128	34	145	108	37	595	566	29
KCY	36	445	390	55	296	245	51	178	110	68	441	388	53
LMC	68	241	205	36	172	134	38	123	88	35	304	268	36
LWS	11	517	449	68	249	176	73	185	132	53	434	380	54
PTM	57	344	310	34	176	131	45	80	52	28	282	244	38
SKW	30	504	480	24	258	226	32	148	111	37	411	375	36
SC	47	258	203	55	324	237	87	116	85	31	277	238	39
TYC	18	327	280	47	135	100	35	125	82	43	255	213	42
TCY	56	238	212	26	190	156	34	103	76	27	313	287	26
WCK	31	552	513	39	88	261	27	100	67	33	500	459	41
MEAN	40.36	425.73	384.64	41.09	209.45	182.64	45.00	132.73	92.27	40.45	413.18	373.64	39.55
S.D.	17.45	197.76	196.10	14.05	72.09	56.14	18.72	33.05	23.09	12.71	150.61	150.19	8.50

3.1.4 Correlation between the pre-operational, post-operational plasma ALP levels and the prognosis of osteosarcoma

After 4 courses of preoperational chemotherapy and at the day before operation, the mean value of the plasma TALP level in the group 1R (osteosarcoma patients with recurrent disease) was 425.73 IU/L, S.D. 72.09 IU/L, while the mean value of the plasma BALP was 182.64 IU/L with S.D. 56.14 IU/L. On the other hand, the mean and S.D. of the plasma TALP and BALP of the group 1N was 151.44 IU/L with S.D. 66.96 IU/L, and 105.78 IU/L with S.D. 69.90 IU/L respectively. The value was summarized in the following

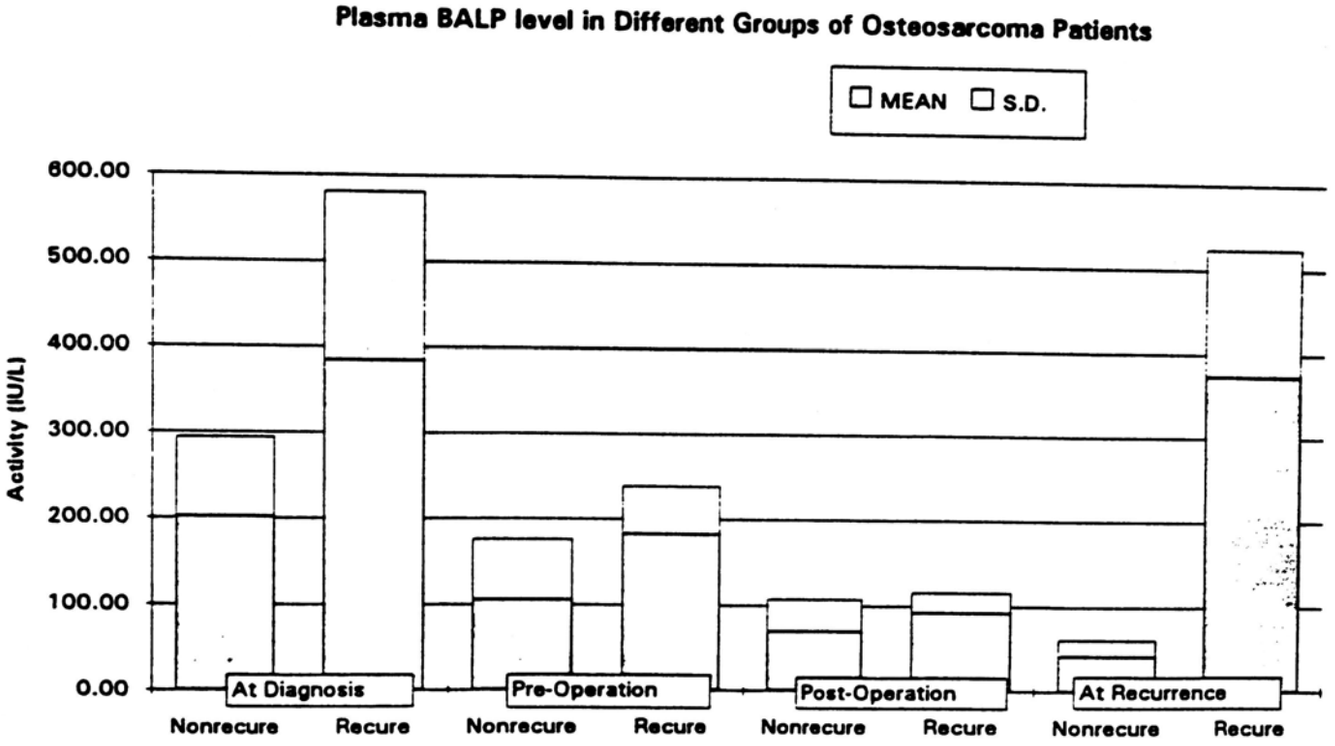


Figure 3-3 Mean and S.D. of the plasma TALP and BALP level in group 1 patients at the time pre-operation and post operation.

When Wilicoxon Matched-Pairs Signed-Ranks Test was performed, both the plasma TALP and BALP level of the group 1R were NOT significantly higher than the corresponding value of the group 1N (osteosarcoma patients without recurrence). In TALP value, Z= -1.7178, 2-Tailed P = 0.0858 which means $p > 0.05$, and BALP value was a little bit better than the TALP value with Z=-1.820 and 2-Tailed P significant = 0.0687, which means still $p > 0.05$.

At the time of post-operation, the mean value of the plasma TALP level in the group 1R (osteosarcoma patients with recurrent disease) was 132.73 IU/L, S.D. 33.05 IU/L, while the mean value of the plasma BALP was 92.27 IU/L with S.D. 23.09 IU/L. On the other hand, the mean and S.D. of the plasma TALP and BALP of the group 1N was 114.22 IU/L with S.D. 35.56 IU/L, and 69.22 IU/L with S.D. 37.86 IU/L respectively. The value was summarised in the figure 3-3, while the details of the individual results was recorded in table 3-5.

Although the mean value of the plasma TALP and BALP level in the patients of recurred osteosarcoma was higher than that of the non-recurred group, but again, when Wilicoxon Matched-Pairs Signed-Ranks Test was performed, both the plasma TALP and BALP level were NOT significantly higher than the corresponding value of the group 1N (osteosarcoma patients without recurrence). In TALP value, Z value = -1.6803, 2-Tailed P = 0.0926 which means p> 0.05, and BALP value was a little bit better than the TALP value with Z = -1.7178, 2-Tailed P = 0.0858, which still p> 0.05. Moreover, the 2-Tailed significant value of the post-operation was even worse than that of the pre-operational value, figure 3-4.

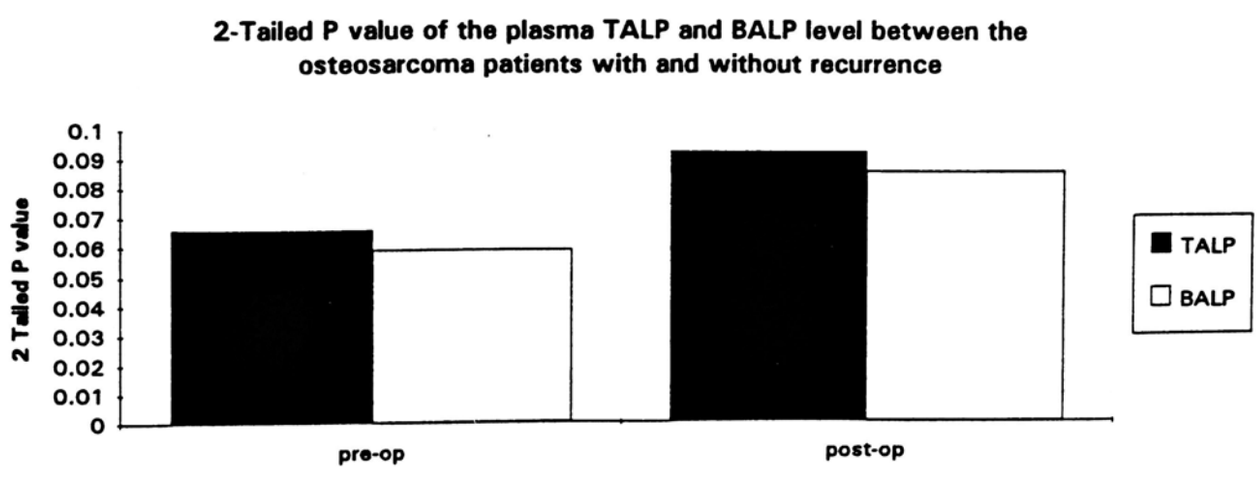


Figure 3-4 2-Tailed P value of the preoperation and postoperation BALP and TALP level

3.1.5 Analysis of plasma ALP levels at the time of relapse in osteosarcoma patients

At the time relapse, the mean value of the plasma TALP level in the group 1R (osteosarcoma patients with recurrent disease) was 413.18 IU/L, S.D. 150.61 IU/L, while the mean value of the plasma BALP was 373.64 IU/L with S.D. 150.19 IU/L. While the mean and S.D. of the plasma TALP and BALP of the group 1N at the time of follow-up clinic was 82.56 IU/L with S.D. 25.14 IU/L, and 41.44 IU/L with S.D. 20.14 IU/L respectively. The value was summarized in the following figure 3-5, while the details of the individual results was recorded in table 3-5.

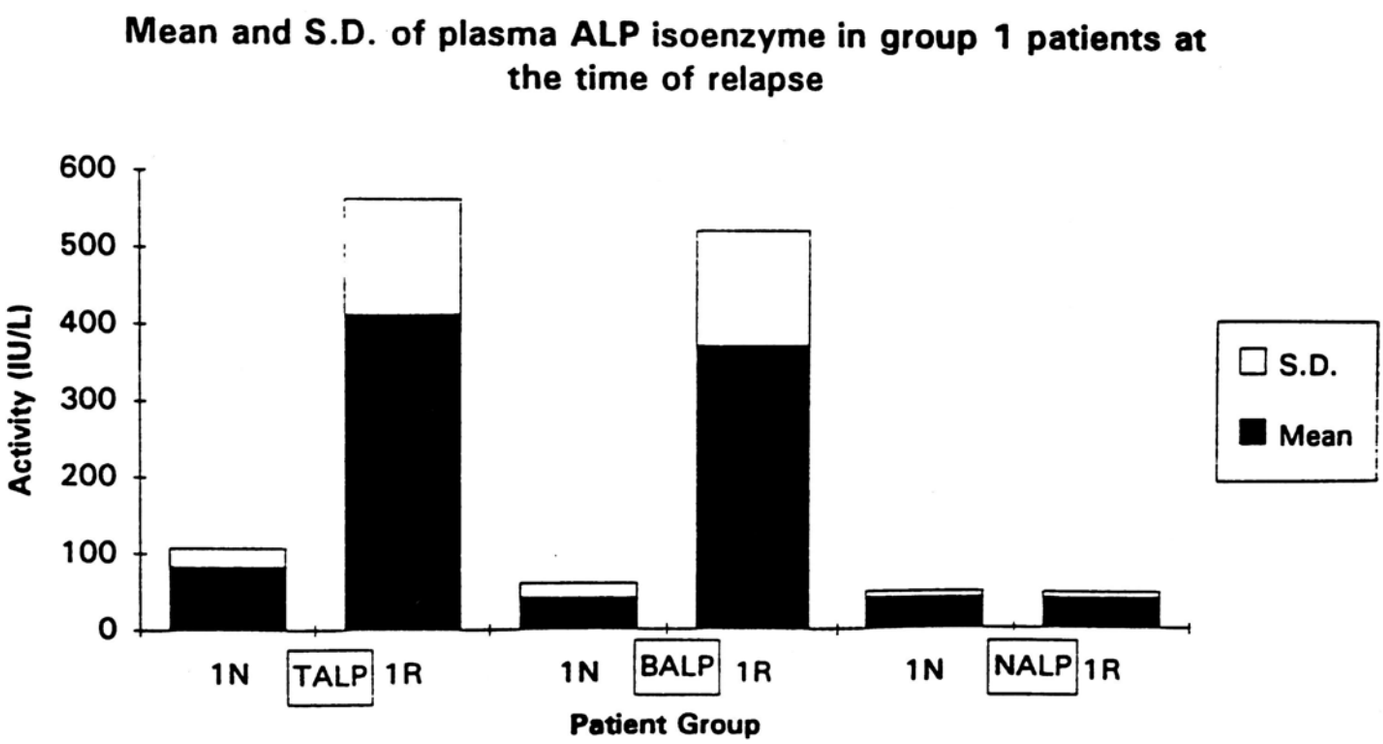


Figure 3-5 Mean and S.D. of the plasma TALP and BALP level in group 1R patients at the time of recurrent and in the group 1N patients at the follow-up clinic.

When Wilicoxon Matched-Pairs Signed-Ranks Test was performed, both the plasma TALP and BALP level were **significantly higher** than the corresponding value of the group 1N (osteosarcoma patients without recurrence). Both the plasma TALP and BALP level has Z = -2.6656, 2-Tailed P significance = 0.0077 which means $p < 0.05$.

Moreover, when the value of the plasma BALP of the group 1R patients at the time of recurrent was compare with the value of the same patient at the time of post-operation, there was **significant increase**. With Z =-2.5471 and 2-Tailed P significance = 0.0109 which means $p < 0.05$.

On the contrary, the values of the plasma BALP of the osteosarcoma patient without relapse of the diseased (group 1N), has **significantly decrease** with the value of the same patient at the time of post-operation. Z value = -2.9341 and 2-Tailed P significance = 0.0033, which means $p < 0.05$.

3.1.6 Usefulness of the plasma ALP levels for monitoring the effectiveness of chemotherapy

In the present study, most patients response to the pre-operational and post-operational chemotherapy. We found that the improvement of clinical symptoms and radiologically decrease of tumor size, was well correlated to the decrease of plasma BALP level of the patients. After each course of chemotherapy, the plasma BALP level dropped. Moreover, after operation, the plasma BALP level also showed a small decrease. The results of the plasma BALP level in the patients was recorded in the figure 3-6 below.

However, there were 4 patients that were not tolerate to the preoperational chemotherapy. One of which, SC, developed acute hepatitis A infection and in cases of LMC and TYC, they had developed severe side effect including vomiting

and febrile reaction and therefore stopped the chemotherapy. The plasma BALP level of these 3 patients with the retread of chemotherapy was increased, which is correlated to the increase of the tumor size with clinical unimprovement of the symptom The last patient, CLY, although have a significantly decrease of plasma BALP level pre-operationally, the fluctuation of the BALP level before each course of chemotherapy showed us that he is also not response to the chemotherapy. The monitoring results of the plasma BALP of the patients that were not tolerated to the chemotherapy was recorded in the figure 3-7 below.

Moreover, at the time of post-operational chemotherapy, the increase of plasma BALP level was latter proved with pulmonary metastasis or local recurrent of the disease. The increase of plasma ALP level at most 3 weeks precede any clinical symptom and 4 weeks precede any radiological evidence of recurrence of the disease.

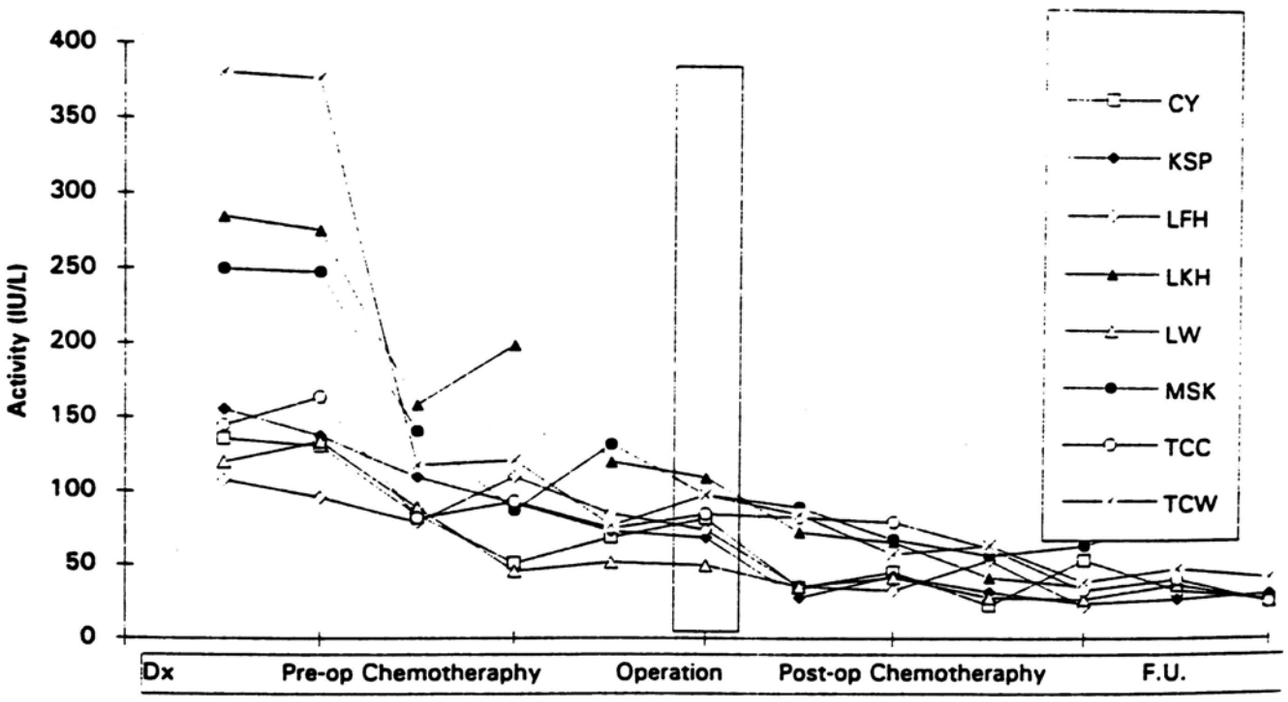


Figure 3-6 *Results of the pre-operational and post-operational plasma BALP level in the osteosarcoma patients that response well to the chemotherapy.*

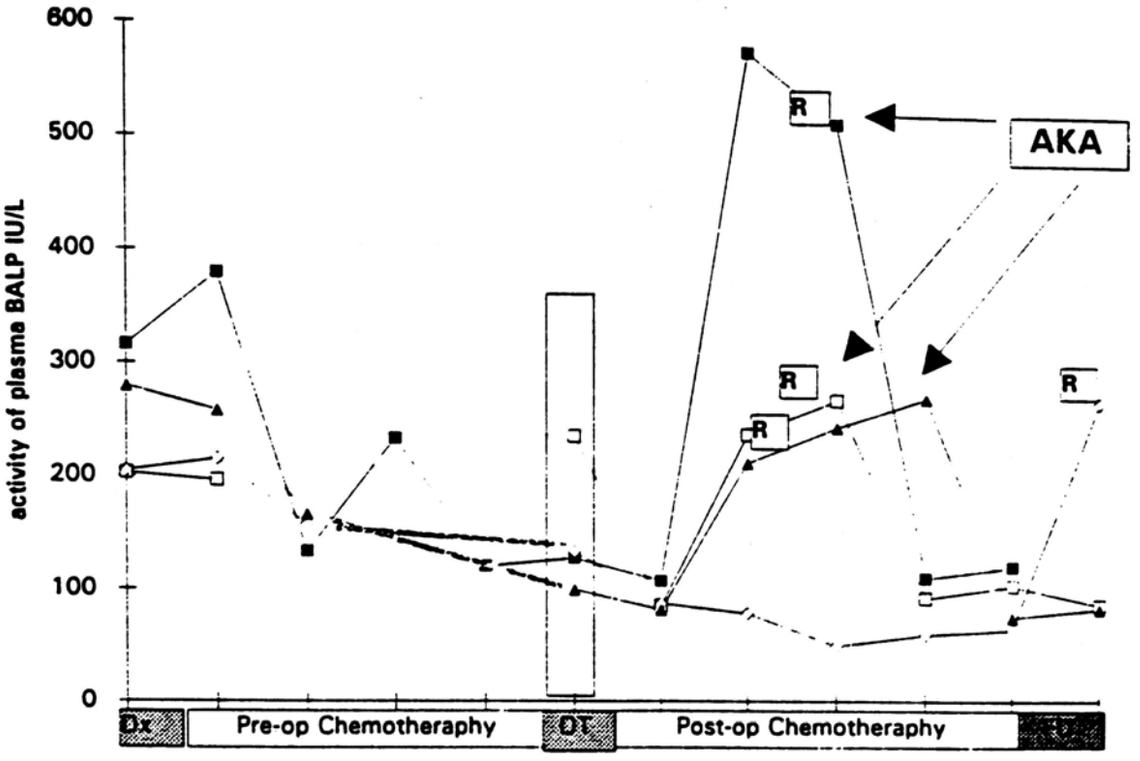


Figure 3-7 Results of the pre-operational and post-operational plasma BALP level in the osteosarcoma patients that were not tolerated to the chemotherapy.

3.1.7 Correlation between the ALP levels in the tumor extract and the prognosis of the osteosarcoma

At the time of Excision and Allograft Operation, both the excised tumor and the normal cortical bone tissue sampled from the illiac bone graft was used for biochemical analysis. The mean value of the tissue BALP level in the group 1R was 115.78 IU/L, S.D. 74.17 IU/L, while the mean and S.D. of the tissue BALP of the normal cortical bone of the same patient was 1.59 IU/L with S.D. 1.10 IU/L. On the other hand, the mean and S.D. of the tissue BALP of the

group 1N and their normal control was 50.26 IU/L with S.D. 35.02 IU/L, and 1.70 IU/L with S.D. 1.06 IU/L respectively. The value was summarized in the following figure 3-8, while the details of the individual results was recorded in table 3-6.

Name	Osteosarcoma Tissue			Normal Cortical Bone		
	TALP	*BALP*	*NBALP*	*TALP*	*BALP*	*NBALP*
CHL	45.58	43.28	2.30	0.86	0.79	0.07
CY	15.96	14.75	1.18	1.20	1.01	0.19
KSP	32.55	31.57	0.98	4.10	3.70	0.40
LFH	23.60	21.80	1.60	0.90	0.80	0.10
LKH	154.42	125.70	28.72	1.79	1.61	0.18
LW	95.39	84.37	11.02	2.41	2.10	0.31
MSK	44.86	41.60	3.26	3.15	3.10	0.05
TCC	48.14	31.37	16.77	1.59	1.22	0.37
TCW	60.20	57.86	2.34	1.18	0.98	0.20
MEAN	57.86	50.26	7.57	1.91	1.70	0.21
S.D.	42.90	35.02	9.60	1.11	1.06	0.13

Name	Osteosarcoma Tissue			Normal Cortical Bone		
	TALP	*BALP*	*NBALP*	*TALP*	*BALP*	*NBALP*
AKM	130.65	111.82	18.83	2.31	2.26	0.05
CLY	159.00	152.00	7.00	1.20	1.01	0.19
KCY	168.44	156.24	12.20	2.41	2.14	0.27
LMC	130.14	123.30	6.84	1.38	0.98	0.40
LWS	46.71	42.57	4.14	3.72	3.42	0.30
PTM	19.29	17.02	2.27	3.15	2.88	0.27
SKW	71.84	67.97	3.87	1.17	1.02	0.15
SC	58.06	56.68	1.38	0.19	0.12	0.06
TYC	76.25	75.38	0.87	0.09	0.08	0.01
TCY	273.87	256.47	17.40	1.43	1.23	0.20
WCK	223.88	214.13	9.75	2.90	2.40	0.50
MEAN	123.47	115.78	7.69	1.81	1.59	0.22
S.D.	78.74	74.17	6.22	1.18	1.10	0.15

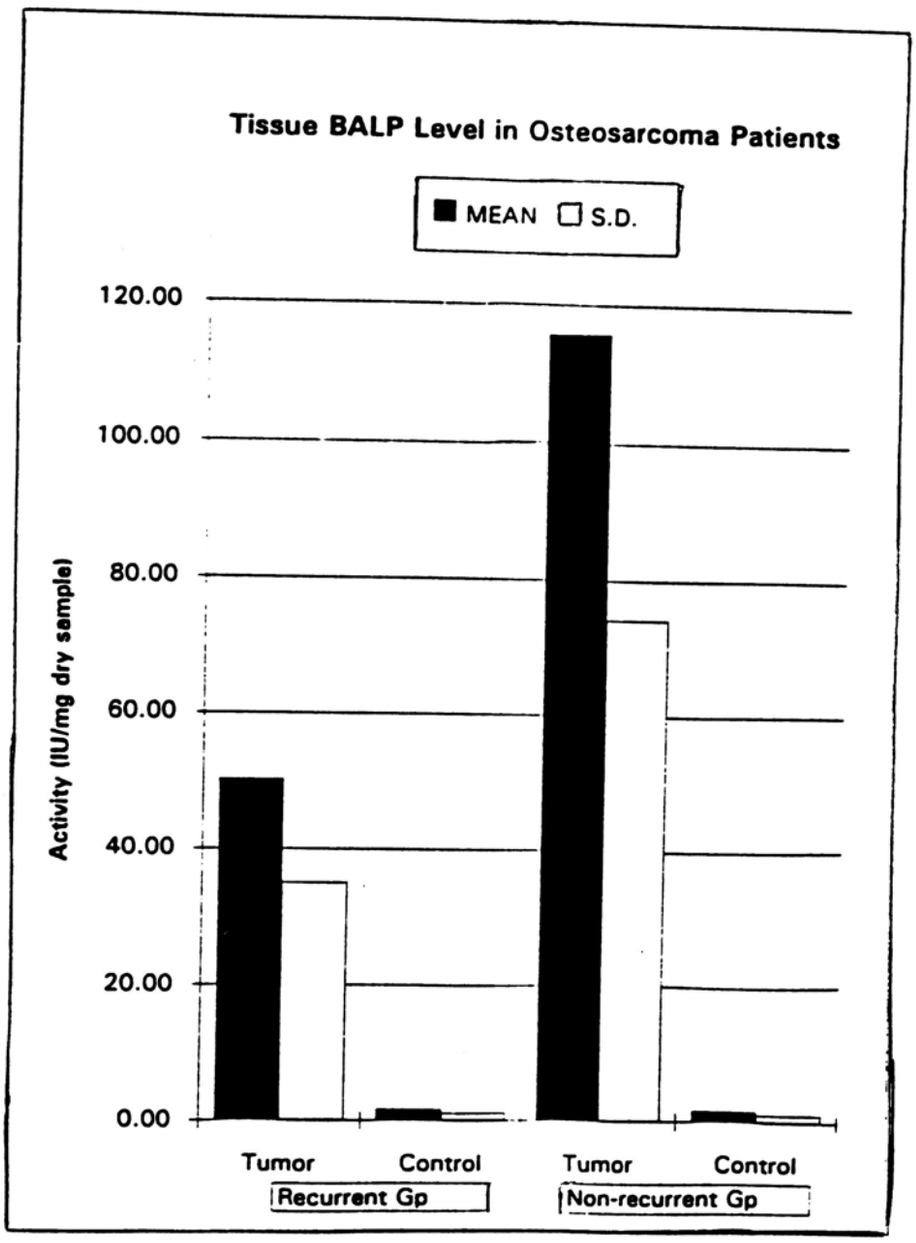
Table 3-6 Mean and S.D. of the BALP activity in the tumor tissue extract and the normal cortical bone tissue extract of the Osteosarcoma patients.

When Wilicoxon Matched-Pairs Signed-Ranks Test was performed, both the tissue BALP level of the recurrent and nonrecurrent group were significantly higher than their normal control, with significance at the level $p< 0.05$. The Z value = -2.6656 2-Tailed P = 0.0077 in group of osteosarcoma patients without recurrence of the disease (1N) and the significant P value was even a little bit better in the group of patients with recurrent osteosarcoma (1R) with Z value = -2.9341, 2-Tailed P = 0.0033.

On the contrarily, there was no significant between the normal control of the two group 1R and 1N, with Z value =-4-0.4201 and 2-Tailed P significance = 0.6744 which means $p> 0.05$.

When we compare the BALP activity of the tumor extract of the two group 1R and 1N, we found that the mean value of the recurrent group was higher than that of the nonrecurrent group, 89.22 IU/mg dry sample to 50.18 IU/mg dry sample. However, when Wilicoxon Matched-Pairs Signed-Ranks Test was performed, there was no significantly different between these 2 group at the level of $p = 0.05$. The Z value was -1.4809 with 2-Tailed P significance = 0.1386 which was larger than the acceptable significant level of $p > 0.05$.

Figure 3-8 Mean and S.D. of the BALP activity of the osteosarcoma tissue and the normal control cortical bone tissue of the same patient.



3.2 QUALITATIVE ANALYSIS OF THE PLASMA AND TISSUE ALKALINE PHOSPHATASE LEVEL

3.2.1 Comparison of the result of Isoelectric focusing of the plasma ALP of the osteosarcoma patients and the normal subjects

The result of the separation of pathological and normal plasma on agarose gel obtained by the isoelectric focusing (IEF) electrophoresis technique was in the following photo 3-1. The first lane (lane 1) is the lane of molecular weight marker stained by the Coomasie Blue R250 staining, which is the staining for protein. The plasma from the venous blood of the normal subject (lane 2), the plasma of the venous blood of the recurrent osteosarcoma patients (lane 3) and that of the osteosarcoma patients without recurrent of disease (lane 4) were stained by the immunological antigen-antibody staining.

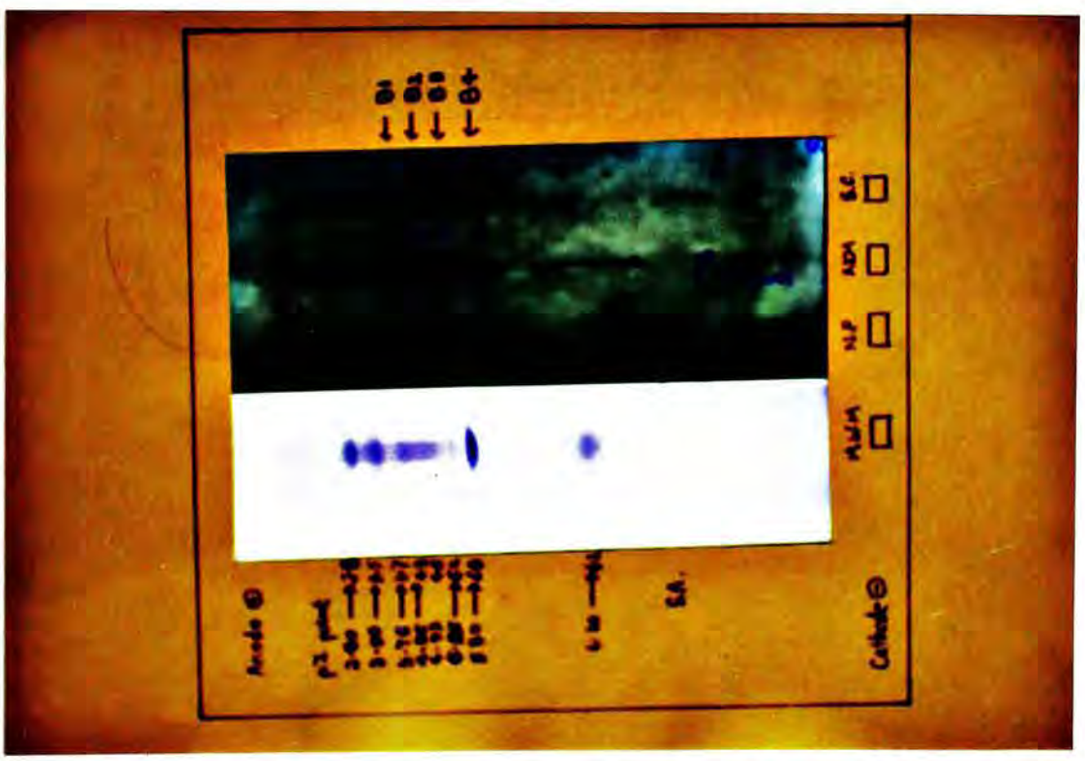


Photo 3-1 The result of the separation of pathological and normal plasma on agarose gel obtained by the isoelectric focusing (IEF) electrophoresis technique. Details of each lane and bands are discribed in the text above.

It is show that in the first lane, the molecular weight marker, 8 bands were obtained. This is correlated to the 8 component inside the molecular weight marker with the pI point of 2.8, 3.5, 3.75, 4.35, 5.20, 5.85 and 6.55 respectively. the result of the IEF gel after Western Blotting process of the normal and pathological plasma was recorded on the following figure 3-9. On the contrary, in every lane of the lane 2 to lane 4, four bands could be found. The B1, B2, B3 and B4 are carrying the pI point of 3.60, 4.30, 4.95 and 5.85 respectively from the anode to the cathode.

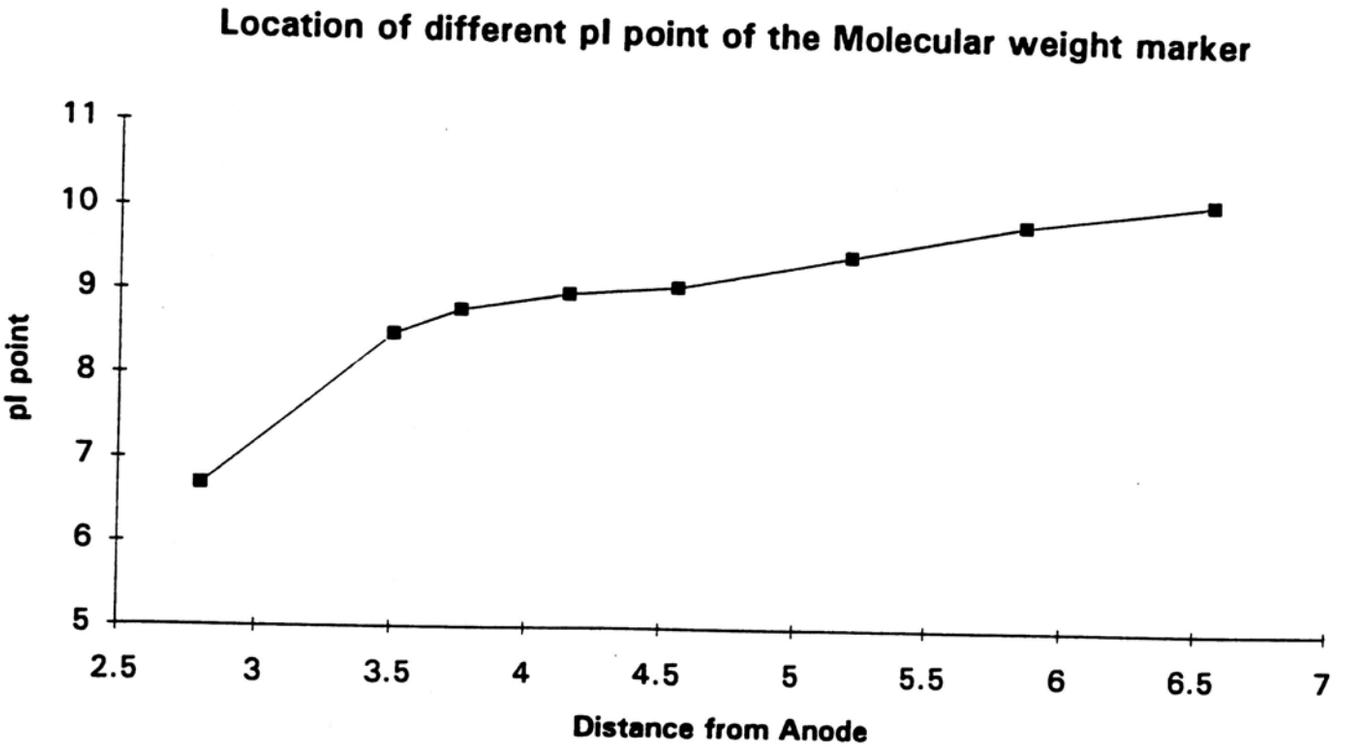


Figure 3-9 The result of the IEF gel after Western Blotting process of the normal and pathological plasma.

On the other hand, the result of the IEF gel after Western Blotting process of the normal and pathological plasma was recorded in the photo 3-2 below. In this gel, which is stained by the Coomasie Blue R250 staining after 1 hour process of Western blotting. The lane 1 is the reference control of the plasma of the osteosarcoma patient which has not been transfer to the membrane while the lane 2 is the plasma of the same patient which has been transfer to the membrane after 1 hour Western blotting. It can be seen that almost all the protein in the plasma had been transferred to the membrane. The lane 3 and 4 this are the plasma of the patient with benign bone tumor and the normal subject respective. In the lane 3 and 4, it could hardly found any bands on the IEF gel. This proved that almost all the protein in the IEF gel was transferred to the cellulose membrane by the Western Blotting process.

Photo 3-2 The result of the IEF gel after Western Blotting process of the normal and pathological plasma.

3.2.1 Result of Isoelectric focusing of the ALP isoenzymes in the tissue extract of the osteosarcoma and normal bone

The photo 3-3 showed the IEF gel of the tissue extract of the osteosarcoma tumor and the normal cortical bone tissue of the same patient. The first lane (lane 1) is the lane of molecular weight marker stained by the Coomasie Blue R250 staining, which has been described before. The second lane (lane 2), was the cord blood which only contain the bone specific ALP isoenzyme. The lane 3 contain the normal bone tissue extract, the lane 4 were obtained from the extract of the osteosarcoma tissue of the same patients and lastly the lane 5 is the same tissue extract sample of lane 4 but previous treated with wheat germ lectin incubation. The lane 2, 3, 4 and 5 were stained by the immunological antigen-antibody staining. It is show that in all the lane 2 to 5 only 2 bands, instead of the 4 bands found in the plasma sample, was obtained.

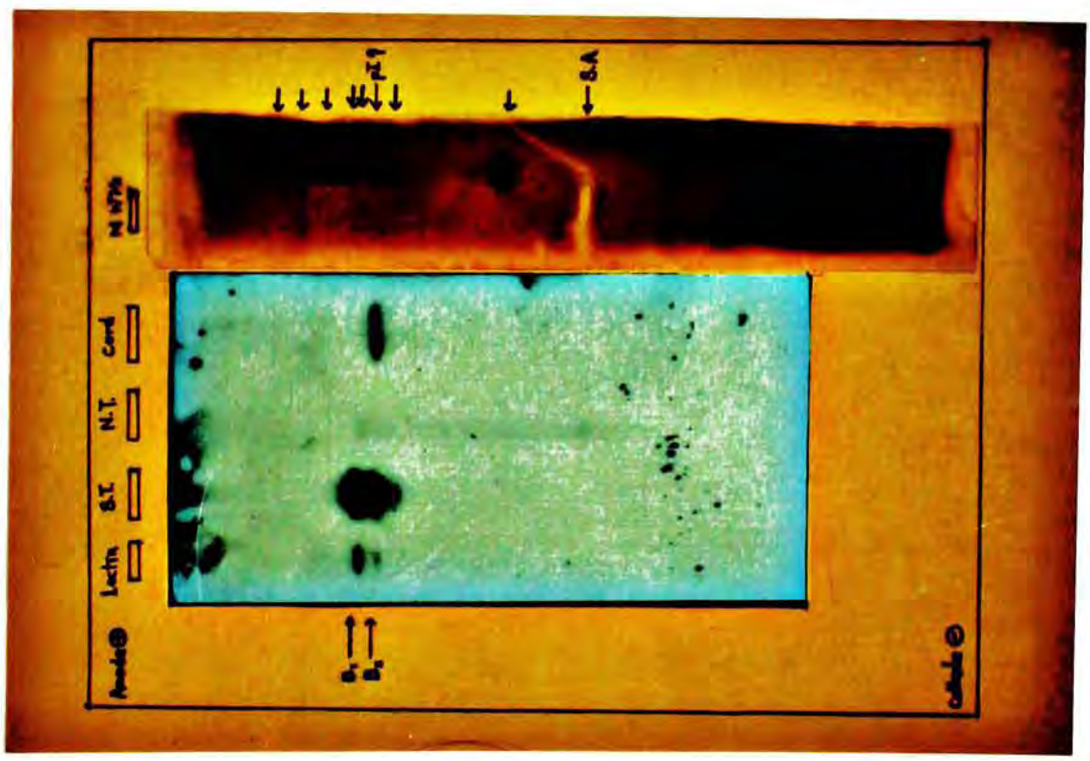


Photo 3-3 The result of the separation of tissue extract on agarose gel obtained by the isoelectric focusing (IEF) electrophoresis technique. Details of each lane and bands are discribed in the text above.

Chapter 4
Discussion

4.1 USE OF QUANTITATIVE MONITORING OF PLASMA ALP AND MEASURING TISSUE ALP IN OSTEOSARCOMA PATIENTS

Alkaline phosphatase is thought to be involved either in the calcification of the bone matrix and in the protein synthesis activity associated with the bone matrix production.[18,79]. Culture osteosarcoma cell from human cell line [22], and animal cell line [24], have been shown to produce large amounts of this enzyme. In the osteosarcoma patients, the disease of abnormally high production of osteoid or immature bone, there is fairly good correspondence between the activity of plasma ALP and the degree of osteoblastic activity of the tumor. Many authors reported that 40% to 80% of the patients with osteosarcoma has abnormally elevated activity of plasma ALP [5,50,70,79,81]. In the first part of the present experiment, we found that in all the 20 patients with osteosarcoma, the plasma ALP level was abnormally high. The mean level of plasma TALP is 343.90 IU/L and that of the plasma BALP is 302.65 IU/L, they are significantly higher than the normal value of plasma TALP, 136 IU/L and the normal value of plasma BALP level, 56 IU/L respectively, at the level of $p<0.001$. We also found that the plasma TALP and BALP level of the group of patients with osteosarcoma is significantly higher than that of the group of patients with benign bone tumor and that of the group of patients with malignant tumor metastasis to the bone, at the significant level $p< 0.05$. Therefore, it is proved that the measuring of both plasma TALP and BALP level is a fairly useful investigation to help diagnose the disease of osteosarcoma. The measuring of plasma ALP is especially useful in the clinically untypical or non-characteristic borderline cases, such as some benign bone tumor, the chondroma, osteoma, the giant cell tumor, or the discovery of the skeletal metastatic site of some other malignant tumor.

In the cases of malignant tumor metastasis to skeletal system, the present study showed that the plasma BALP level was not significantly higher than that of the normal value at the level p equals 0.05. The result was support by the previous work of Dr. K.S. Leung in our laboratory [49] including 38 patients with osteolytic skeletal metastasis of the malignant tumor. However, some previous authors[61] found that the level of plasma ALP was elevated in the patients of skeletal deposit of the malignant tumor. The variation may be explained by the fact that what they measured was the plasma total ALP (TALP) instead of the bone specific isoenzyme of ALP (BALP) used in our study. In the present experiment, the plasma TALP level of the patients with skeletal metastasis of the malignant tumor (group 3 patients) had a very high variation, which reflected by the high S.D. value of 172.42 IU/L which is even high than the mean value of the plasma TALP level of 114.58 IU/L. The large variation is due to the cases of hepato-carcinoma which had a very high level of NBALP, actually the hepatic isoenzyme of the plasma ALP, while the bone specific isoenzyme (BALP) remain normal. On the contrary, the plasma BALP level had a more accurate and smaller S.D. value of 8.17 IU/L. Therefore, we suggest that plasma bone specific isoenzyme of the alkaline phosphatase (BALP) should be measured instead of the plasma total alkaline phosphatase (TALP) level.

With the simple lectin precipitation method introduced by Rosalki and Foo, we can accurately measure the bone specific isoenzyme of the alkaline phosphatase (BALP). The present study found that in the plasma of the patients with osteosarcoma, the ratio of plasma BALP to TALP is above 85 % which is significantly higher than that of the normal subjects, 40 %, and that of the patients with benign bone tumor and skeletal metastasis of malignant tumor. This is another usefulness of measuring plasma BALP which specifically reflected the osteoblastic activity of the osteosarcoma disease.

As we discussed before, with the introduction of aggressive adjuvent chemotherapy, the long term survival rate of osteosarcoma patients has recently be improved. As a consequence, its now possible to evaluate the prognostic factor of the long term survivor. Among the prognostic factors previously analysed by other researchers, the sex and age of the patients, the length of the pre-diagnostic clinical symptoms, the tumor size, the tumor site, radiological signs or even the pathological cell type of the osteosarcoma are of uncertain value [45,66,70,86]. So as in the present study, the above factor contributed very small the prediction of the prognosis of the disease of osteosarcoma. In the present study, subdivided the group of osteosarcoma patient into 2 subgroups, the group with recurrent of disease during the period of monitoring (1R), and the group of patients remain nonrecurre of the disease (1N) during the period of monitoring. When we compare the pretreatment level of plasma ALP, both the level of TALP and BALP of the group of patients with recurrent disease of osteosarcoma (1R) is significantly higher than that of the nonrecurre group (1N) at the level of $p<0.05$ (2-Tailed P significance = 0.0209). The result was supported by the previous work of Thorpe et al.[79] from the National Cancer Institute at Bethesada (NCI), they conclude that pretreatment plasma ALP level could be a prognostic factor in osteosarcoma: the higher the ALP levels, the higher the rate of relapse. G. Bacci in Rizzoli [5], which a larger group of 163 osteosarcoma patients also support our finding. Price in 1987 [91] proved that with the increase of mitotic activity of the osteosarcoma cell, the rate of relapse was increase. As we discussed before, the increase of cell activity and the increase of the production of bone matrix need the presence of ALP. Therefore, the osteosarcoma with higher the osteoblastic activity, which reflected by the higher the level of plasma ALP, will have higher chance of relapse.

Moreover, the hypothesis of micrometastasis theory[61,87] presume that all the patients without any evidence of metastasis of the disease of osteosarcoma developed micrometastasis or invisible deposit of the tumor cell through the entire body. Therefore, with higher the level of plasma ALP, the more the spreading of the microscopic tumor cell, the shorter the period of relapse and the worse the prognosis.

In the present study, the level of the plasma ALP isoenzyme of the patients at the period after 4 course of preoperational chemotherapy but before the surgical treatment (preoperational) was measured. Moreover, the result of the postoperative plasma ALP isoenzyme activity was also compared. We found that both the preoperational and postoperative plasma BALP and TALP of the group of patients with recurrent osteosarcoma (1R) had a higher mean level than that of the nonrecure group (1N). However, the Wilicoxon Matched-Pairs Signed-Ranks Test proved that there was no significant difference between the 2 groups of patients at p=0.05 level. In addition, when we compare the 2-Tailed P significance of the two group, the p value of the preoperational plasma ALP level is better (smaller) than that of the postoperative plasma ALP level. The result was summarized in the following table 4-1. This may be explained by the increase of variational factors, such as the factors of the different methods of operative treatment, different scheme of chemotherapy and the false increase due to the Kuntscher rod or the autograft, therefore, the relationship of the postoperative serum ALP level was not so significant as that of the preoperational plasma ALP values. With the more the variation factors, the worse the significant value of the plasma ALP activity. Besides, the 2-Tailed P significance of the plasma BALP level was better than that of the plasma TALP level, both at the time of preoperation and postoperaton. This further proved the advance of plasma BALP to the TALP in monitoring the disease of osteosarcoma.

Time	Isoenzyme	Mean Value		2-Tailed P value
		Recurre (1R)	*Nonrecure (1N)*	
Diagnosis	TALP	425.73 IU/L	248.33 IU/L	0.0209
	BALP	384.64	202.44	0.0209
Preop.	TALP	209.45	154.14	0.0858
	BALP	182.64	105.78	0.0687
Postop.	TALP	132.73	114.22	0.0926
	BALP	92.27	69.22	0.0858
Relapse or follow-up	TALP	413.18	82.56	0.0077
	BALP	373.64	41.44	0.0109

Table 4-1 The 2-Tailed P Significance of the plasma BALP and TALP level of the osteosarcoma patients with (1R) and without recurrence (!N) of the disease, at the time of diagnosis, preoperational (Preop.), postoperational period (Postop.), and at the time of relapse or at the regular follow-up clinic.

The studies of many previous authors proved that the serum ALP activity raised to an abnormal level at the time of recurrent or metastasis[5,53]. Moreover, the raise of serum ALP preceded by several weeks the appearance of symptom and clinical sign, and before the detection of metastasis by x-ray. In the present study, the plasma ALP level of the group of patients at the time of recurrence, is significantly higher than that of the group of patients without the evidence of recurrence at the time of follower-up clinic. Moreover, the level of the plasma ALP is significantly increased from that recorded at the time of postoperation of the same patients. In addition the increase of plasma ALP level precede the clinical evidence of the relapse of the disease by 3 weeks and precede any radiological evidence of recurrence.

This proved that plasma ALP can be a good indicator and should be included in the monitoring of the osteosarcoma patients. Futhermore, measuring of plasma ALP is a more simple and less invasive investigation than the radiological investigation, or histopathological results which required the technique of open biopsy.

As it was proved by the study of Bacci et al.[5] that the lack of response of the level of plasma ALP after the operative treatment, 100% of the patients relapsed in a shorter period of time. We found that all the 20 patients has a significant decrease of plasma BALP level after the operative treatment. This showed that the Excision and Allograft operation of the osteosarcoma patients is a proper and effective treatment. Besides, in the present study that the patients who response to the chemotherapy, had a clinical improvement of the symptoms and the radiological evidence of decrease of tumor size showed corresponding decrease of plasma BALP level. While in the group of patient tolerate to the chemotherapy, with increase of tumor size and recurrence of the clinical symptoms, their plasma ALP level was increased. This proved that the level of plasma BALP is a good indicator reflecting the osteobalstic activity of the osteosarcoma, it can be used to show the effectiveness of the chemotherapy. With the increase of plasma BALP level during the period of chemotherapy, Oncologists should be alerted of the torlerance of the therapy, increase of drug doses or changing of the other regime or even go directly to the operational treatment should be consider.

When the result of BALP level of the osteosarcoma tissue extract was analysed, the present study showed that it was significantly higher than that of the normal cortical bone tissue sampled from the same patient, at the significant level $p< 0.005$. Moreover, the tissue needed for the biochemical assay is as little as 50 mg, it might be possible to get the tissue even at the time of biopsy.

Therefore, the present study proved that measuring the BALP level in the tumor tissue might be a useful tool helping diagnosing the disease of osteosarcoma. The result was supported by previous work of Alan M. Levine[44] that with the activity of the sarcoma tissue extract was significantly higher than the normal bone tissue. He also reported that with the activity of the sarcoma tissue extract higher than 0.6 umol/min/mg tissue (600 IU/gm tissue), the time for relapse of the osteosarcoma was shorter. However, in the present study, although we found that the mean level of BALP in the tumor extract of the patients with recurrence of the disease, 115.78 IU/gm dry tissue, is higher than that of the patients with nonrecurre disease, 50.18 IU/gm dry tissue, the Wilicoxon Matched-Pairs Rank Signed Test showed that there is no significant difference between the 2 groups of patients at the level $p < 0.05$. It may be explained by the fact that the different regime of the postoperational chemotherapy and the difference of the operative treatment affect the significance of the BALP activity of the tissue extract.

Table 4-2 The 2-Tailed P Significance of the BALP and TALP level of the osteosarcoma tumor tissue extract of the patients with and without recurrence of the disease..

Moreover, when the result of the tumor tissue BALP level of the present study is compared with that of the previous work done by Alan M. Levine, the mean BALP level in the tumor extract of the present study was 87.63 IU/gm dry tissue which is lower than the mean level, 202 IU/mg dry tissue, Levine's report. The variation might be due to the different method used to detect the ALP activity of the tissue extract, Levin used electrophoresis and substrate method which was different to our lectin precipitate method.

The ALP activity we measured in the tissue extract was the BALP level, and the NBALP portion of the tissue extract is very small and can be neglected. In the discussion of the latter section, the qualitative analysis of the plasma and tissue ALP activity, it was proved by the method of isoelectric focusing electrophoresis technique (IEF) that the bone tissue or the osteosarcoma tissue only contain band of the bone specific portion of the ALP.

4.2 ISOELECTRIC FORCUSING AS A TECNIQUE FOR QUALITATIVE MEASUREMENT OF PLASMA AND TISSUE ALKALINE PHOSPHATASE

Using IEF on agarose gel with a pH gradient from pH 3.0 to pH 10, we have routinely detact 4 distinguishable bands of ALP activity in the plasma of the healthy subjects and in the plasma of osteosarcoma patients. As any technique that detects multiple bands of enzyme activity, two essential questions must be answered: Do the bands represent true ALP activity? What is the cellular origin of the multiple bands in the normal or pathological subjects?

The use of immunological antigen-antibody staining in the present study answere the first question affirmatively. The antisera was specifically bind to the ALP isoenzyme or isoform, therefore, no other enzyme or protien other than the ALP can be stained by the present method. The second question may be approached by studying the composit picture derived from the normal and

pathological plasma and that of the molecular weight marker. The relative position of each band can be estimate through the known value of the molecular weight marker. The four observed band, B1, B2, B3 and B4 in lane 2 to 4 of photo 3-1 are at the same distance from the anode. Therefore, the plasma of the normal subject and the plasma of the 2 osteosarcoma patients are consider of having the same composition of the type of isoenzymes. The pI point of the band B1, B2, B3 and B4 of the normal and patient plasma are 3.60, 4.30, 4.95 and 5.85 respectively. By comparing to the results found by the previous authors[27,74,90], we proved the band B1 and B2, is the liver and bone isoenzyme respectively. Moreover, as discribed by the previous author, the cord blood contain only the bone specific ALP. The only one band found in the IFE gel of cord blood (lane2 of photo 3-3) carrying the pI point 4.30. This futher proved that the band 2, is the bone specific ALP isoenzyme.

The intensity of the band 2 is obviously higher in the plasma of the patients with osteosarcoma, the lane 3 and 4 of the photo 3-1, than that of the band 2 in normal plasma (lane 2). This is agree with the quantitative measurement of the plasma ALP isoenzyme level in the first part of the present study.

When the result of the tissue extract of the osteosarcoma tissue and the normal bone extract (photo 3-3) is studied, no band can be found in the normal bone tissue (lane 2) and only 2 bands can be obtained from the tumor tissue extract (lane 4). The invisible of any bands in the lane 2 showed that the activity of the normal bone tissue extract is too low to be detected by the present IEF electrophoresis technique. After calculation and comparing with the molecular weight marker, the band found in tumor tissue (lane 4) carry the pI point of 4.15 in the first band (B1) and pI point equals 4.30 in the second band (B2). The second band (B2) is at the same position as the only band found in the cord blood (lane 2) which is equals 4.30. This showed that the band 2 found in the

tissue extract is the bone specific ALP isoenzyme, which is the same as that found in the plasma in the photo 3-1. The detect of the more anodal minor band (B1) is agree with the previous work of Moss et al [57] in 1982. The presence of minor zone result from the formation of the aggregation of other proteins and lipids with the hydrophobic domains of the ALPs, which located in the plasma membrane of the cell, leading to a larger sizes of the molecules, thus retarding migration in the starch gel. Moreover, after the treatment with wheat germ lectin (lane 5 of photo 3-3), the B2 band of the lane 5 is obviously reduced. This proved that the bone specific ALP isoenzyme of the tissue extract is inhibited by the treatment of wheat germ lectin. This is agree with the result of the quantitative measure of the bone specific ALP, by the Rosalki's wheat germ lectin percipitated method, in the first part of the present study.

In the present study, agarose gel was used as the supporting media for isoelectric forcusing of the alkaline phosphatase isoenzyme of the plasma and tumor tissue extract. It was suggested by the prevous researchers that agraorse gel is better than the polyacrylamide gel that smaller cathode shift is found in the agarose gel. Besides, the larger pore size of the agarose gel allow more protein molecules to pass through, this is more fruitful when the unknown size of the protein is going to be detected. Sinha [74] and some author[27,90] suggest that 1-2-naphthyl phosphates as substrate, coupled to a diazolium salt was responsible for difused and blurred bands in conventional IEF, therefore, in the present study, indoxyl phosphatase-tetrazolium salt combination has been used. Four bands are observed in the plasma and two bands are observed in the bone tumor tissue extract. However, by the method used in this study, no abnormal extra-band, the tumor related or tumor marker can be discovered either in the plasma or in the tissue extract of the sarcoma patient. Therefore, a more detail method, the 2-Dimansion gel technique should worth trying in the futher study.

Chapter 5
Conclusion

Conclusion

The present study concluded that the measurement of bone specific isoenzyme of the alkaline phosphatase (BALP) of the plasma and tumor tissue extract is a useful investigation for the diagnosis of osteosarcoma. Besides, the pretreatment level of the plasma BALP is a good predicting factor of the prognosis of the disease. Moreover, plasma BALP activity can also be used to show the effectiveness of the chemotherapy and can be used to detect the relapse of the osteosarcoma. Therefore, we suggest the routine measurement of plasma BALP level in every case of suspected osteosarcoma, and should be included in the follow-up clinic. In addition, the qualitative measurement of the plasma and tumor tissue of the osteosarcoma patient proved that the increased portion of the ALP isoenzyme is the bone specific isoenzyme, however, there is not any abnormal band found by the present electrophoresis technique.

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